1-1-2003

Interactions among arbuscular mycorrhizal fungi and their impact on soybean growth

Satoshi Ishii
Iowa State University

Interactions among arbuscular mycorrhizal fungi and their impact on soybean growth

by

Satoshi Ishii

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Soil Science (Soil Microbiology and Biochemistry)

Program of Study Committee:
Thomas E. Loynachan, Major Professor
Larry J. Halverson
Thomas C. Harrington

Iowa State University

Ames, Iowa

2003

Graduate College
Iowa State University

This is to certify that the master's thesis of

Satoshi Ishii

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy

TABLE OF CONTENTS

CHAPTER 1. LITERATURE REVIEW: FUNCTIONAL IMPORTANCE OF ARBUSCULAR MYCORRHIZAL FUNGAL DIVERSITY IN NATURAL ECOSYSTEMS 1

Introduction 1
Methods to Detect AM Fungal Species from the Environment 8
Research in AM Fungal Diversity 13
Functional Complementarity in AM Fungal Diversity 16
Future Prospective 20
References 22

CHAPTER 2. RAPID, RELIABLE, AND INEXPENSIVE DNA EXTRACTION TECHNIQUES FROM TRYPAN BLUE-STAINED MYCORRHIZAL ROOTS 34

Abstract 34
Introduction 35
Materials and Methods 38
Results 44
Discussion 48
References 51
Appendix 54

CHAPTER 3. DIFFERENT COLONIZATION RATES OF AN ARBUSCULAR MYCORRHIZAL FUNGUS BETWEEN TWO SOYBEAN CULTIVARS 56

Abstract 56
Introduction 57
Materials and Methods 58
Results 60
Discussion 65
References 68
Appendix 70

CHAPTER 4. INTERACTIONS AMONG THREE ARBUSCULAR MYCORRHIZAL FUNGI AND THEIR IMPACT ON SOYBEAN GROWTH 73

Abstract 73
Introduction 74
Materials and Methods 76
Results 85
Discussion 106
References 114
Appendix 118

ACKNOWLEDGMENT 120

CHAPTER 1. LITERATURE REVIEW: FUNCTIONAL IMPORTANCE OF ARBUSCULAR MYCORRHIZAL FUNGAL DIVERSITY IN NATURAL ECOSYSTEMS

Introduction

Overview

Arbuscular mycorrhizal (AM) fungi have strong effects on plant productivity, plant biodiversity, and other microorganisms in the mycorrhizosphere. These characteristics are, however, largely different at the genus and sometimes at the species level, and this difference might cause AM fungal diversity observed in natural ecosystems. In this review, we discuss general topics in these AM fungal effects, their differences among AM fungal species, and recent progress in the study of AM fungal diversity. Functional differences in AM fungi might explain the several conflicting findings on AM fungal effects in nature.

General introduction to arbuscular mycorrhizal fungi

More than 80% of terrestrial plants associate with arbuscular mycorrhizal (AM) fungi (Smith and Read, 1997), because AM fungi, being in the order Glomales, have broad host ranges that enable them to colonize with many plants. This broad host specificity of AM fungi might contribute to maintain AM fungal diversity in soil (van Tuinen et al., 1998).

AM fungi have been classified based on their spore morphology into three families, *Acaulosporaceae*, *Gigasporaceae*, and *Glomaceae*, but recent molecular as well as morphological data revealed two new families, *Archaeosporaceae* and *Paraglomaceae* (Morton and Redecker, 2001). Phylogenetic analysis of the five families of AM fungi based on the 18 S rRNA gene sequences showed that the same families were clustered in the same group, except the *Glomaceae* (Schwarzott et al., 2001). Since functional differences are often discussed based on taxonomy (Hart and Klironomos, 2002; Hart and Reader, 2002; Klironomos and Hart, 2002), it is important to reexamine the generic concept within AM fungi (Schwarzott et al., 2001).

Impact on plant productivity

General benefit for plants is that they can receive more minerals, especially the nutrients with poor mobility such as P and Zn, through mycorrhizal association (Smith and Read, 1997). Khalil et al. (1994) reported that AM fungi promoted corn and soybean growth by improving plant P and N uptake. AM fungi also have been reported to improve plant water relations in a semiarid ecosystem (Sanchez-Diaz and Honrubia, 1994; Requena et al., 1996, 2001). Therefore, AM fungi have a strong influence on plant productivity. These topics were well reviewed by Smith and Read (1997).

Negative effects of AM fungi, however, have been also documented (Abbott and Robson, 1985; Thomson et al., 1986, 1990; Jakobsen et al., 1992a; Pearson and Jakobsen, 1993a; Koide, 1995; Johnson et al., 1997). Several factors such as developmental, environmental, and genotypic factors could affect AM fungal function to be negative or positive (Johnson et al., 1997). AM fungal colonization can decrease growth of plant

seedlings few wk after planting (Abbott and Robson, 1985; Koide, 1985; Thomson et al., 1986, 1990; Jakobsen et al., 1992a; Johnson et al., 1997). Also, positive and negative effects of AM fungi might be partially explained by the concept of preferable host-fungal combinations based on complementary/redundant function between AM fungal hyphae and plant roots (Koide, 2000), which is discussed later in this chapter. Functions of AM fungi along the continuum from mutualism to parasitism were reviewed by Johnson et al. (1997).

More recently, some AM fungi were reported to associate simultaneously with their host plants and with non-photosynthetic plants, termed epiparasites (Bilartondo et al., 2002). Epiparasitic plants obtain carbohydrate from photosynthetic plants through mycorrhizal association (Bilartondo et al., 2002; Hibbett, 2002). Therefore, AM fungi may indirectly cause growth depression of their host plants through association with myco-heterotrophs (Hibbett, 2002).

Impact on plant biodiversity

AM fungi may also have a strong influence on plant biodiversity (Grime et al., 1987; Gange et al., 1990; Streitwolf-Engel et al., 1997; van der Heijden et al., 1998a, b; Klironomos et al., 2000; van der Heijden et al., 2002). Klironomos et al. (2000) reported that AM fungi clearly affected plant community structure. They showed, as the "generalized niche model" (Tilman et al., 1997) predicted, that the greater the diversity of plant communities, the better they can use the limiting resources. They observed that the existence of AM fungi changed the relationship between plant biodiversity and ecosystem productivity from a positive and linear relationship to a positive and asymptotic relationship, presumably because AM fungi help plants with their nutrient acquisition. Plants can better utilize

limiting nutrients with AM fungi, resulting in more ecosystem productivity and richness in small plant species and, therefore, an asymptotic relationship between plant biodiversity and ecosystem productivity develops (Klironomus et al., 2000). There are conflicting results, however, that decreased mycorrhizal activity increased plant biodiversity (Hartnett and Wilson, 1999; O'Connor et al., 2002), suggesting the effect of AM fungi on plant biodiversity is different among host and/or fungal species (Bergelson and Crawley, 1988; Hartnett and Wilson, 1999; O'Connor et al., 2002).

AM fungi also significantly influence plant reproduction; therefore they have long-term effects on plant community structures (Sanders and Koide, 1994; Poulton et al., 2002). Important interactions between AM fungi and plant populations were reviewed by Sanders et al. (1998) and Koide and Dickie (2002). Relationship between diversity of AM fungi and ecosystem function was also reviewed by Hart and Klironomos (2002). A review article on the impact of AM fungi on plant diversity is also available (van der Heijden, 2002).

Impact on microbial community structure

AM fungi influence not only plant biodiversity, but also bacterial community structure of the mycorrhizosphere. Mycorrhizosphere is defined as the soil zone influenced by mycorrhizal roots (Linderman, 1988). Rhizosphere bacterial community structure is different between mycorrhizal and non-mycorrhizal roots (Secilia and Bagyaraj, 1987; Kothari et al., 1991; Christensen and Jakobsen, 1993; Posta et al., 1994; Andrade et al., 1997; Fillion et al., 1999; Marshner et al., 2001). Others also have observed the effect of AM fungi on other bacterial and fungal species. Previous reports have shown that infection of AM fungi affects bacterial population size in the rhizosphere (Bagyaraj and Menge, 1978; Ames

et al., 1984; Meyer and Linderman, 1986; Posta et al., 1994; Marschner et al., 2001) and their rate of growth (Christensen and Jakobsen, 1993; Marschner and Crowley, 1996). Relationships between AM fungi and other soil organisms were more thoroughly reviewed by Gryndler (2000).

AM fungi have been reported to protect plants from some pathogenic diseases (Schőnbeck, 1979; Dehne, 1982; Newsham et al., 1995; Green et al., 1999; Bødker et al., 2002; Yao et al., 2002). Bødker et al. (2002) reported that AM fungi affected not the vegetative stage but the spore-forming stage of the pea pathogen, *Aphanomyces euteiches*. Green et al. (1999) suggested suppressive effect of *Glomus intraradices* against the fungal soil-borne plant pathogen, *Trichoderma harzianum*, was due to nutrient competition. The system of suppression by AM fungi, however, is still unclear. Suppressive and conductive soils against *Ralstonia solanacearum*, which causes wilt disease for solanaceous plants such as tomato, have different microbial community structures (Shiomi et al., 1999); therefore one possibility is that AM fungi mediate the microbial community and prevent the pathogen from growing dominantly. Effects of AM fungi on plant disease suppression were reviewed in detail by Linderman (2000) and Sylvia and Chellemi (2001).

Different impacts among AM fugal species

These impacts described above are different among AM fungal species. Van der Heijden et al. (2002) observed that three different *Glomus* isolates influenced differently plant biomass, plant P content, plant N content, and root and hyphal length per pot. Other researchers have reported the difference in the ability to acquire nutrients among AM fungi by using radioactive ^{33}P, stable ^{32}P and/or stable ^{15}N isotopes (Jakobsen et al., 1992a, b;

Pearson and Jakobsen, 1993a, b; Ravniskov and Jakobsen, 1995; Smith et al., 2000). Colonization strategies such as means of infection, infection speed, colonization rates within plant roots, and hyphal length in soil are different among AM fungal species (Hart and Reader, 2002; Klironomos and Hart, 2002), suggesting different impact of AM fungi on their host plant. Recent molecular approaches have revealed the different influence of AM fungi on plant gene expressions regulating the P starvation response (Burleigh et al., 2002). Molecular approaches to investigate the interaction between AM fungi and plants were well reviewed by Harrison (1999), Franken and Requena (2001), Barker et al. (2002), and Barker and Larkan (2002).

Other reports have shown that mycorrhizal dependency varied significantly among plant species (Hetrick et al., 1988; Koide and Li, 1991) and even among cultivars (Khalil et al., 1994; 1999). Khalil et al. (1994; 1999) observed that different corn and soybean cultivars have shown different dependency to the two kinds of AM fungi (*Gigaspora margarita* and *Glomus intraradices*), indicating plants have some preference to their companion AM fungi. Streitwolf-Engel et al. (1997) reported that three different AM fungi (all belonging to the genus *Glomus*) showed different effects on the clonal growth traits of two grassland plant species. They verified that the differences among AM fungi were not due to their colonization rate; therefore they proposed that the species richness of AM fungi might contribute to plant population structure. Van der Heijden et al. (1998b; 2002) suggested that different plant species profited from different AM fungi to different extents based on their greenhouse and field experiments. These varying effects of AM fungi imply one reason why AM fungal diversity contributes to the maintenance of plant populations in natural ecosystems (van der Heijden et al., 1998a, b, 2002).

Microbial community structure of the rhizosphere varies also among plant roots infected by different mycorrhizal fungi (Secilia and Bagyaraj, 1987; Paulitz and Linderman, 1989; Krishnaraj and Sreenivasa, 1992; Andrade et al., 1997; Marshner et al., 2001). Marshner et al. (2001) reported that the bacterial community structure varied between two rhizosphere environments infected by the different AM fungi after 7 wk, although they were similar after 4 wk. The differences in exudate composition and/or amount from mycorrhizal roots might be one reason for the differences in bacterial community structure (Linderman, 1988; Dixon et al., 1989; Paulitz and Linderman, 1989; Kothari et al., 1991; Waschkies et al., 1994), growing the concept of the myrorrhizosphere (Linderman, 1988). AM fungal species-specific effect on bacteria in the rhizosphere has been documented (Secilia and Bagyaraj, 1987; Paulitz and Linderman, 1989; Krishnaraj and Sreenivasa, 1992; Marschner and Crowley, 1996; Andrade et al., 1997), supporting the finding that the microbial community structures were different between the mycorrhizosphere infected by the different AM fungal species (Marschner et al., 2001).

Therefore, AM fungal diversity might be important to maintain plant biodiversity, plant ecosystem productivity, and microbial community structure in the rhizosphere.

Methods to Detect AM Fungal Species from the Environment

Several methods to detect AM fungi

It is almost impossible to identify AM fungi colonizing within plant roots at the species level by using conventional and morphological techniques. Morphological characteristics of some AM fungi vary in different host plant root systems. Therefore, methods to detect AM fungal species *in planta* have been developed.

Several methods have been applied to detect AM fungi *in planta*. Antibodies (Hahn et al., 1993), isozyme patterns (Hepper et al., 1988; Thingstrup and Rosendahl, 1994), lipid profiles (Bentivenga and Morton, 1994; Madan et al., 2002), and molecular genetic methods have been used. Hepper et al. (1988) used isozyme patterns to detect AM fungi in plant roots. The advantage of this method is that it makes possible detecting metabolically active fungi, but its detection sensitivity is different among fungal species (Hepper et al., 1988). Lipid profiles have potential as a biomass indicator for AM fungi but do not distinguish at the AM fungal species level (Madan et al., 2002).

Recently, many researchers have applied polymerase chain reaction (PCR)-based molecular techniques to analyze AM fungi at the species level. Usually, ribosomal RNA genes (rDNA) are used because they contain both highly conserved regions throughout the organisms and specific regions to each species. Researchers have used 18 S rDNA (Simon et al., 1992, 1993; Clapp et al., 1995; Di Bonito et al., 1995; Helgason et al., 1998, 1999; Chelius and Triplett, 1999; Schwarzott et al., 2001 Schwarzott and Schüßler, 2001; Vandenkoornhuyse et al., 2002), large subunit (25 – 28 S) rDNA (van Tuinen et al., 1998;

Clapp et al., 2000; Jacquot et al., 2000; Turnau et al., 2001), and 5.8 S rDNA and internal transcribed spacer (ITS) regions between 18 S and large subunit rDNA (Sanders et al., 1995; Redecker et al., 1997; Millner et al., 1998; Hijri et al., 1999; Redecker et al., 1999; Redecker, 2000; Millner et al., 2001a, b). 18S rDNA has evolved relatively slowly and therefore their sequence variability is not high enough to characterize AM fungi at the species level (Chelius & Triplett, 1999). ITS regions, however, have more variable sequences among AM fungal species (Millner et al., 2001a, b; Sanders et al., 1995; Redecker et al., 1997; Redecker, 2000). Redecker (2000) designed PCR primers specific to AM fungal sub-groups and his contribution enables us to characterize AM fungal species more easily. D2 domain of large subunit rDNA also provides sufficient information to distinguish AM fungi at the species level (van Tuinen et al., 1998). Therefore, these genes are useful to identify AM fungi in a community.

PCR-based techniques

Several PCR-based techniques have been applied to the community analyses of AM fungi. Random amplified polymorphic DNA (RAPD, also called arbitrary primed PCR) has proven to be useful to distinguish isolates within individual species and possibly among species if enough primers are used (Wyss and Bonfante, 1993; Lanfranco et al., 1995). This approach is more useful when specific primers or probes are to be designed and target DNA sequences are unknown.

Second-step PCR (nested PCR) with species-specific primers in their second reaction is a simple and reliable technique to characterize AM fungal species in plant roots (van Tuinen et al., 1998; Jacquot et al., 2000; Turnau et al., 2001). Specific oligonucleotide

probes also have a possibility to be a strong tool to characterize AM fungi in plant root systems (Sanders et al., 1998), but it requires a lot of work to design both species-specific primers and specific oligonucleiotide probes. Although several species-specific PCR primers and specific oligonucleotide probes have been established (Abbas et al., 1996; Millner et al., 1998, 2001a, 2001b; van Tuinen et al., 1998), they cover just a few species of AM fungi. Therefore, neither species-specific nested-PCR nor specific oligonucleotide probes have been broadly used for the study of AM fungal diversity. Once species-specific PCR primers and/or specific oligonucleotide probes are established, these techniques may show a great advantage.

Restriction fragment length polymorphism (RFLP) of PCR-amplified rDNA has been used to show the diversity of AMF within plant roots (Helgason et al., 1998; Vandenkoornhuyse et al., 2002b). PCR-RFLP was verified useful to distinguish AM fungus at the species- and sometimes, even the strain-level (Sanders et al., 1995; Redecker et al., 1997; Redecker, 2000). Single stranded conformation polymorphism (SSCP) of PCR-amplified rDNA has been tested for usefulness for AM fungal diversity study (Simon et al., 1993; Kjøller and Rosendahl, 2000). Amplified fragment length polymorphism (AFLP) of AM fungal DNA was also applied to study genetic variation intra- and inter-species of AM fungi (Rosendahl and Taylor, 1997). Although, it has not applied in AM fungal studies, denaturing gradient gel electrophoresis (DGGE) is a good tool to analyze microbial communities (Muyzer et al, 1993). DGGE has been used to assess fungal community structures (Kowalchuk et al., 1997; Pennanen et al., 2001). Because SSCP, AFLP, and DGGE can detect more polymorphism than RFLP, they have possibility to become useful tools to detect species using shorter PCR fragments than those in RFLP.

Sequencing cloned DNA is the most reliable technique to identify species. Sequencing bands of interest in RFLP analysis might be useful for minimizing labor and cost (Vandenkoornhuyse et al., 2002b). Based on sequenced DNA, species-specific PCR primers or oligonucleotide probes can be designed for further ecological study in AM symbiosis.

Application and possible problems of PCR-based techniques

These DNA-based molecular techniques can be applicable to analyze AM fungal diversity in soil as well. Methods to extract microbial DNA from soil have been developed by many researchers (e. g. Claasen et al., 1996; Nazar et al., 1996; van Elsas and Smalla, 1996; Chelius and Triplett, 1999). Claasen et al. (1996) and Chelius and Triplett (1999) developed an AM fungal DNA extraction method from soil samples, and it can facilitate the AM fungal diversity study in soil. Study of AM fungal diversity outside plant roots has been little examined because of the inability of microscopy to observe AM fungal structures in soil, but development of molecular techniques can solve this problem. Because extent of AM fungal colonization in soil is different from that in plant roots (Hart and Reader, 2002), AM fungal colonization in soil should be studied as well as colonization within roots.

A possible problem of PCR-based molecular techniques is that the amount of PCR product may not reflect species abundance. Helgason et al. (1999), however, showed by comparing between extensive morphological work and the PCR-based estimates of AM fungal relative proportion, that it might be possible to measure species composition and abundance quantitatively by PCR-based techniques if the sequence types are sampled enough. Quantitative-PCR was tested for its usefulness as a tool to quantify AM fungal abundance within plant roots (Edwards et al., 1997). Also, combination of microscopy and PCR-based

techniques was applied to measure the relative proportion of AM fungi within plant roots (Jacquot et al., 2000). Terminal restriction fragment length polymorphism (T-RFLP) is a quantitative DNA fingerprinting technique (Liu et al., 1997; Blackwood et al., 2003) with more sensitivity than DGGE (Moeseneder et al., 1999). Dickie et al. (2002) used T-RFLP to characterize ectomycorrhizal hyphae in soil, and therefore, it can be useful to study complex AM fungal communities as well. Development of a reliable and quantitative method is one of the current issues for AM fungal community analyses.

Another problem of PCR-based species identification is that contaminated fungal DNA might be amplified, especially when DNA is extracted from spores (Clapp et al., 2002a, b). Single spores of AM fungi have been reported to contain highly divergent rRNA gene sequences (Clapp et al., 1995; Sanders et al., 1995; Lloyd-MacGilp et al., 1996; Clapp et al., 1999; Sanders, 1999; Hijri et al., 1999; Antoniolli et al., 2000; Pringle et al., 2000; Clapp et al., 2001; Rodriguez et al., 2001; Jansa et al., 2002). AM fungi are coenocytes, which means many nuclei exist within the same cell wall (Sanders, 1999), and this might derive different sequences with a single spore (Sanders, 1999). Although variation exists among species, one spore of AM fungi generally contains around 600-4000 nuclei (Hosny et al., 1998), and these nuclei are not necessarily homogeneous. Since conjugations and nuclear exchanges among AM fungal hyphae were observed *in vivo* (Giovannetti et al., 2001); different nuclei might exist in hyphae and in a single spore (Sanders, 1999). However, we could not exclude the possibility of amplification of contaminant DNA (Schüßler, 1999). High divergence, including several families, might also be caused by high variability within the ITS region (Schüßler, 1999). In the case of *S. castanea*, one genome contained 75 ± 10 copies of rRNA gene unit and these genes were heterogenous (Hosty et al., 1999). There is, however,

controversy on the finding of Hosty et al. (1999); Schüßler (1999) and Redecker et al. (1999) disputed that highly divergent ITS copies were originated from contaminated Ascomycete.

Since even cleaned single spores contain plant, animal, or ascomycetes DNA (Schüßler, 1999) and almost all families of fungi were detected from plant roots (Vandenkoornhuyse et al., 2002a), we have to be very careful in identifying DNA sequences obtained from these samples and prevent mislabeling of a contaminant as AM fungi (Clapp et al., 2002a, b).

Molecular identification of AM fungi and genetic studies of AM fungal communities were reviewed by Redecker (2002) and Clapp et al. (2002a), respectively.

Research in AM Fungal Diversity

Diverse spore detection from natural ecosystems

In natural ecosystems, different AM fungal spores have been detected (Hetrick et al., 1994; Johnson et al., 1992; Troeh and Loynachan, 2003). They observed the diversity of AM fungal spores has changed along with their host plant. Although spore production of AM fungi does not always reflect root colonization (Clapp et al., 1995), existence of different AM fungal spores indicates that diversity of AM fungi does exist in natural ecosystems.

Effect of mixed inoculation on plants

The effects of mixed inoculation have been documented. Previous reports showed that plants grow much better with various kinds of AM fungi than with a single species of

AM fungi (van der Heijden et al., 1998a). Their greenhouse and field experiments suggested that the diversity of AM fungi might cause a great increase in the length of mycorrhizal hyphae in the soil, and contributed to the plant nutrient uptake. Moreover, they observed a positive relationship between AM fungal diversity and plant biodiversity. Although their findings significantly contributed to the study of AM fungal diversity, they did not examine AM fungal differences in the rate and the extent of colonization within plant roots. To verify their suggestion that different plant species profited from different AM fungi to different extents, the colonization rate and extent of each AM fungi in plant roots should be examined.

Coexistence of AM fungi in plant roots

Many studies have reported that multiple AM fungal species coexist within the same root system (Wilson and Trinick, 1983; Wilson, 1994; Rosendahl et al., 1989; McGonigle and Fitter, 1990; Clapp et al., 1995; van Tuinen et al., 1998; Jacquot et al., 2000). Van Tuinen et al. (1998) showed that all four fungi they studied (*Glomus mosseae*, *G. intraradices*, *Gigaspora rosea*, and *Scutellospora. castanea*) were detected within the same root fragment by species-specific nested PCR. They also reported the colonization of *Gig. rosea* and *S. castanea* was greatly increased by the presence of *G. mosseae* and *G. rosea* in leek and onion roots but, at the same time, they stated that the result of fungal interactions may depend on the host plant. Similar observations were reported by Jacquot et al. (2000) in that *G. mosseae* colonized roots far better with the coexistence of *G. intraradices* and *Gig. rosea* than when alone. However, suppressions in colonization of some AM fungi with the existence of other AM fungi have also been documented (Wilson and Trinick, 1983; Abbott and Robson, 1984; Wilson, 1984; Hepper et al., 1988; Lopez-Aguillon and Mosse, 1987;

Sainz et al., 1989; Daft, 1993). Hepper et al. (1988) reported that three AM fungi within the genus *Glomus* competed with each other and as a result, one fungus excluded another from the root system. Pearson et al. (1993a) reported that as the amount of inoculum of *S. calospora* increased, both the rate and extent of colonization of *Glomus* sp. decreased. They also suggested plants may mediate the competition between the two AM fungi on a physiological basis such as carbohydrate supply.

Most researchers who reported the existence of AM fungal diversity in the plant roots used molecular techniques to detect AM fungi, but in contrast, those who reported the negative effects among AM fungal species used methods other than molecular techniques. More research is necessary to address the interaction among AM fungal species in the same plant roots.

Field study for AM fungal diversity

Development of molecular genetic techniques enables us to study AM fungal diversity in natural ecosystems. Vandenkoornhuyse et al. (2002) extracted AM fungal DNA from field grassland roots, amplified the DNA by PCR using specific primers to most AM fungi (AM1), and classified the PCR products by their RFLP patterns. Each RFLP pattern was sequenced and divided into several subgroups. Their research revealed the existence of high AM fungal diversity in natural grassland ecology. Similar observations have been reported (Clapp et al., 1995; Daniell et al., 2001; Helgason et al., 1998, 1999; van der Heijden et al., 1998a, b). A summary of AM fungal diversity studies to present shows: 1) the AM fungal diversity in natural ecosystems is high; 2) the AM fungal community is different among different host plants; and 3) the AM fungal community varies by season.

The difference in AM fungal community among their host plants suggests the existence of preferable AM fungi-plant combinations. Seasonal variation in AM fungal community probably varies along with the growth of their host plants; strongly growing plants can provide more carbohydrate to AM fungi, resulting in more colonization in summer than winter, as shown by Daniell et al. (2001).

Functional Complementarity in AM Fungal Diversity

Functional complementarity/redundancy

As previously reviewed, AM fungal diversity does exist within plant root systems. Then, the following questions quickly arise. Why does AM fungal diversity exist? Do these diverse AM fungal species occupy different niches? Many studies support that mycorrhizal impacts on plant productivity, plant biodiversity, and other microorganisms are significantly different among AM fungal genera and/or species. Functional diversity in AM fungal studies can be described as the differences in AM fungal effect on plant responses to both biological and non-biological stress (Smith and Read, 1997) and also on plant growth responses (Burieigh et al., 2002). Diverse functions of AM fungi from positive to negative effects were well reviewed by Johnson et al. (1997).

These functional diversities might be one reason why AM fungal diversity benefits plants: AM fungi might complementarily help plants with their nutrient acquisition (Koide, 2000; Smith et al., 2000), maintain plant biodiversity, and protect plants from disease agents. Hyphal length and ability to acquire P were different between the two AM fungi,

Fig. 1-1. Functional complementarity/redundancy between AM fungal hyphae. AM fungi with different hyphal length colonize in B, and their function in nutrient and water acquisition might complement each other. AM fungi with similar hyphal length colonize the same roots in A and C; therefore their function might be redundant. These figures were originally drawn by Koide (2000). Used with permission.

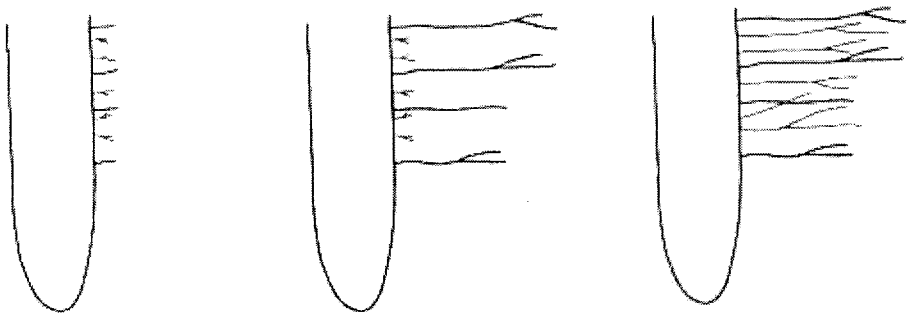
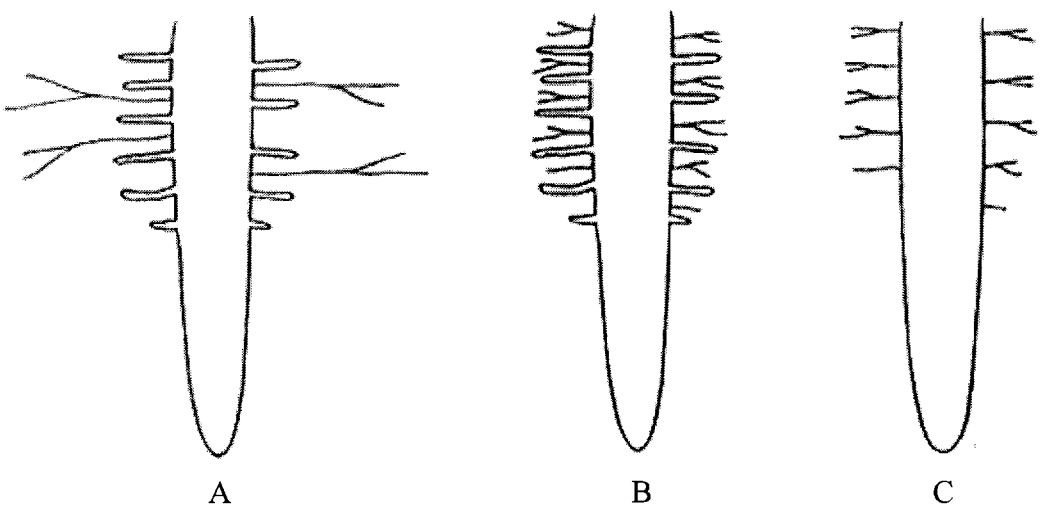


Fig. 1-2. Functional complementarity/redundancy between AM fungal hyphae and plant roots. The function of AM fungal hyphae and plant roots in nutrient and water acquisition might complement each other in A and C, but redundant in B. These figures were originally drawn by Koide (2000). Used with permission.



Scutellospora calospora and *Glomus caledonium* (Pearson and Jakobsen, 1993a, b; Smith et al., 2000). Therefore, if AM fungi with different hyphal lengths and different nutrient acquisition efficiencies are infected together in the same plant, their functions could complement each other (Fig. 1-1) (Koide, 2000). The negative effect of AM fungi in natural ecosystems can be explained by functionally redundant between AM fungal hyphae and plant root hairs (Fig. 1-2), although parasitism of AM fungi also can be explained by developmental factors, environmental factors, and host-fungus combinations (Johonson et al., 1997).

Preferable host-fungus combinations

Preferable host-fungus combinations probably exist because some AM fungi act negatively for certain kinds of plants, while they are beneficial for other plants (Johnson et al., 1992; Johnson et al., 1997; Koide, 2000; Smith et al., 2000). Mycorrhizal dependency varies among plants (Hetrick et al., 1988; Habte and Manjunath, 1991; Koide and Li, 1991; Khalil et al., 1994, 1999) and AM fungal effects also differ among plant hosts (Streitwolf-Engel et al., 1997; van der Heijden et al., 1998a, b, 2002). Preferable combinations of plants and AM fungi can be explained by functional complementarity/redundancy between AM fungal hyphae and plant roots (Fig. 1-2; Koide, 2000). AM fungal species have different abilities to acquire nutrients (Smith et al., 2000) and so do plant species. Different abilities of plants for P uptake might be due to different lengths and densities of plant root hairs, varying capacity to acquire P from insoluble P or organic compound, and diverse aptitude to grow in nutrient-rich areas in soil (van Ray and van Diest, 1979; Barber, 1982; Marschner et al., 1987; Cambell et al., 1991; Tadano et al., 1993). If the AM fungi can acquire P far from the area

that plant root hairs can absorb, this mycorrhizal association functionally complements the plant nutrient acquisition mechanisms (Fig. 1-2A; Koide, 2000). AM fungi having shorter hyphae, however, may compete with plant root hairs in P acquisition, resulting in functional redundancy (Fig. 1-2B).

Preferable host-fungal combinations may cause selection of AM fungi under certain plant communities. Likewise, plant species may be pressured under selection by capability to associate with their favorable AM fungi. This hypothesis supports the finding by van der Heijden et al. (1998a, b, 2002) that AM fungal diversity contributes to the maintenance of plant populations in natural ecosystems. Also, existence of preferable host-fungus combinations and selection of AM fungi by host plants might be one of the reasons why there are both positive and negative interactions among AM fungi colonizing within the same roots (Daft, 1993; Abbott and Robson, 1984; Lopez-Aguillon and Mosse, 1987; Hepper et al., 1988; Sainz et al., 1989; van Tuinen et al., 1998; Jacquot et al., 2000). If some AM fungi are preferred by host plants, they can grow better within plant roots and consume more carbohydrates than others, resulting in a negative effect to other AM fungi. If there is more than one fungus preferred by host plants, it means that they have a complementary function to each other and also to plant root hairs. In this case, plants can acquire more nutrients from soil and can provide more carbohydrates to AM fungi, resulting in positive interactions among AM fungi. Pearson et al. (1993) suggested that a difference in carbohydrate supply by host plants is probably one reason of both positive and negative interactions among AM fungi in the same plant root system. More research is necessary to state the functions of AM fungal diversity and their consequences.

Future Prospective

Existence of high AM fungal diversity in natural ecosystems has been supported by several approaches including recently developed molecular techniques. However, the impact and function of AM fungal diversity on plants and other organisms are still unclear. Diversity of AM fungi might be important in plant productivity and plant biodiversity because preferable host-fungus combinations might exist and work complementarily or redundantly (Koide, 2000). It has not been reported but AM fungal diversity is probably important to maintain microbial community structures because of the different response of microbial community to different AM fungal species. Impact of AM fungi on plant disease suppression should be studied as well as on microbial community structure because disease suppression and microbial community structure might be closely related (Shiomi et al., 1999).

Molecular genetic techniques are useful to detect AM fungi not only within plant roots but also in soil, although quantification of AM fungi in plant roots still has some improvement needed. Molecular techniques based on rRNA genes enable us to identify AM fungi in the environment, but we have to be very careful in species identification based on DNA-based techniques since single spores contain divergent sequences (Clapp et al., 2002b). AM fungal colonization in soil should be more thoroughly studied because the extent of AM fungal hyphae in soil is different among species and fungal biomass in soil does not reflect AM colonization within plant roots (Hart and Reader, 2002). Molecular techniques using the genes coding for specific functions, such as phosphatase activities, facilitate our study in AM fungal function. New methods using radio-active ^{33}P to study AM fungal function in natural

ecosystems were recently developed (Johnson et al., 2001). These techniques enable us to study further AM fungal functions.

Functional roles of AM fungi and their diversity are still unclear, especially in natural ecosystems. Within the coming few years, many findings should come in this field of study.

References

Abbas, J.D., B.A.D. Hetrick, and J.E. Jurgenson. 1996. Isolate specific detection of mycorrhizal fungi using genome specific primerm pairs. Mycologia 88:939-946.

Abbott, L.K., and A.D. Robson. 1984. Colonization of the root system of subterranean clover by three species of vesicular-arbuscular mycorrhizal fungi. New Phytol. 96:275-281.

Ames, R.N., C.P.P. Reid, and E.R. Ingham. 1984. Rhizosphere bacterial population responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. New Phytol. 96:555-563.

Andrade, G., K.L. Mihara, R.G. Linderman, and G.J. Bethlenfalvay. 1997. Bacteria from rhizosphere and hyphosphere soils of different arbuscular mycorrhizal fungi. Plant Soil 192:71-79.

Antoniolli, Z.I., D.P. Schachtman, K. Ophel-Keller, and S.E. Smith. 2000. Variation in rDNA ITS sequences in *Glomus mosseae* and *Gigaspora margarita* spores from a permanent pasture. Mycol. Res. 104:708-715.

Bagyaraj, D.J., and J.A.Menge. 1978. Interaction between a VA mycorrhizal and *Azotobacter* and their effects on rhizosphere microflora and plant growth. New Phytol. 80:567-573.

Barber, S.A. 1982. Soil-plant root relationships determining phosphorus uptake. *In* A.Scaife (ed.) Plant nutrition. Commonwealth Agricultural Bureaux, Warwick.

Barker, S.J., S. Duplessis, and D. Tagu. 2002. The application of genetic approaches for investigation of mycorrhizal symbioses. Plant Soil 244:85-95.

Barker, S.J., and N.J. Larkan. 2002. Molecular approaches to understand mycorrhizal symbioses. Plant Soil 244:107-116.

Bentivenga, S.P., and J.B. Morton. 1994. Stability and heritability of fatty acid methyl ester profiles of glomalean endomycorrhizal fungi. Mycol. Res. 98:1419-1426.

Bergelson, J.M., and J.M. Crawley. 1988. Mycorrhizal infection and plant species diversity. Nature (London) 334:202.

Bidartondo, M.I., D. Redecker, I. Hijri, A. Wiemken, T.D. Bruns, L. Domínguez, A. Sérsic, J.R. Leake, and D.J. Read. 2002. Epiparasitic plants specialized on arbuscular mycorrhizal fungi. Nature (London) 419:389-392.

Blackwood, C.B., T. Marsh, S.-H. Kim, and E.A. Paul. 2003. Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Appl. Environ. Microbiol. 69:926-932.

Bødker, L., R. Kjøller, K. Kristensenm, and S. Rosendahl. 2002. Interaction between indigenous arbuscular mycorrhizal fungi and *Aphanomyces euteiches* in field-grown pea. Mycorrhiza 12:7-12.

Burleigh, S.H., T. Cavagnaro, and I. Jakobsen. 2002. Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. J. Exp. Bot. 53:1593-1601.

Cambell, B.D., J.P. Grime, and J.M.L. Mackey. 1991. A trade-off between scale and precision in resource foraging. Oecologia (Berlin) 87:532-538.

Chelius, M.K., and E.W. Triprett. 1999. Rapid detection of arbuscular mycorrhizae in roots and soil of an intensively managed turfgrass system by PCR amplification of small subunit rDNA. Mycorrhiza 9:61-64.

Christensen, H., and I. Jakobsen. 1993. Reduction of bacterial growth by a vesicular-arbuscular mycorrhizal fungus in the rhizosphere of cucumber (*Cucumis sativus* L.). Biol. Fertil. Soils 15:253-258.

Claasen, V.P., R.J. Zasoski, and B.M. Tyler. 1996. A method for direct soil extractrion and PCR amplification of endomycorrhizal fungal DNA. Mycorrhiza 6:447-450.

Clapp, J.P., J.P.W. Young, J.W. Merryweather, and A.H. Fitter. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol. 130:259-265.

Clapp, J.P., A.H. Fitter, and J.P.W. Young. 1999. Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, *Scutellospora* sp. Mol. Ecol. 8:915-921.

Clapp, J.P., A. Rodriguez, and J.C. Dodd. 2001. Inter- and Intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. New Phytol. 149:539-554.

Clapp, J.P., T. Helgason, T.J. Daniell, and J.P.W. Young. 2002a. Genetic studies of the structure and diversity of arbuscular mycorrhizal fungal communities. p. 202-224. *In* M.G.A. van der Heijden, and I.R. Sanders (ed.) Mycorrhizal ecology. Springer, Berlin.

Clapp, J.P., A. Rodriguez, and J.C. Dodd. 2002b. Glomales rRNA gene diversity - all that glistens is not necessarily glomalean? Mycorrhiza 12:629-670.

Daft, M.J. 1983. The influence of mixed inoculation on endomycorrhizal development. Plant Soil 71:331-337.

Daniell, T.J., R. Husband, A.H. Fitter, and J.P.W. Young. 2001. Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbial Ecol. 36:203-209.

Dehne, H.W. 1982. Interactions between vesicular-arbuscular mycorrhizal fungi and plant pathogens. Phytopath. 72:1115-1118.

Di Bonito, R., M.L. Elliott, and E.A. Des Jardin. 1995. Detection of an arbuscular mycorrhizal fungus in roots of different plant speices wih the PCR. Appl. Environ. Microbiol. 61:2809-2810.

Dickie, I.A., B. Xu, and R.T. Koide. 2002. Vertical niche differentiation of ectomycorrhizal hyphae in soil as shown by T-RFLP analysis. New Phytol. 156:527-535.

Dixon, R.K., H.E. Garrett, and G.S. Cox. 1989. Boron fertilization, vesicular-arbuscular mycorrhizal colonization and growth of *Citrus jambhiri* Lush. J. Plant Nutr. 12:687-700.

Edwards, S.G., A.H. Fitter, and J.P.W. Young. 1997. Quantification of an arbuscular mycorrhizal fungus, *Glomus mosseae*, within plant roots by competitive polymerase chain reaction. Mycol. Res. 101:1440-1444.

Edwards, S.G., J.P.W. Young, and A.H. Fitter. 1998. Interactions between *Psudomonas fluorescens* biocontrol agents and *Glomus mosseae*, an arbuscular mycorrhizal funus, within the rhizosphere. FEMS Microbiol. Lett. 166:297-303.

Fillion, M., M. St-Arnaud, and J.A. Fortin. 1999. Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere micro-organisms. New Phytol. 141:525-533.

Franken, P., and N. Requena. 2001. Analysis of gene expression in arbuscular mycorrhizas: new approaches and challenges. New Phytol. 150:517-523.

Gange, A.C., V.K. Brown, and L.M. Farmer. 1990. A test of mycorrhizal benefit in an early successional plant community. New Phytol. 115:85-91.

Giovannetti, M., P. Fortuna, A.S. Citernesi, and S. Morini. 2001. The occurence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytol. 151:717-724.

Green, H., J. Larsen, P.A. Olsson, D.F. Jensen, and I. Jakobsen. 1999. Suppression of the biocontrol agent *Trichoderma harzianum* by mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* in root-free soil. Appl. Environ. Microbiol. 65:1428-1434.

Grime, J.P., J.M.L. Mackey, S.H. Hiller, and D.J. Read. 1987. Floristic diversity in a model system using experimental microcosms. Nature (London) 328:420-422.

Gryndler, M. 2000. Interaction of arbuscular mycorrhizal fungi with other soil organisms. p. 239-262. *In* Y. Kapulnic, and D.D. Douds Jr. (ed.) Arbuscular mycorrhizas: physioogy and function. Kluwer Academic Publishers, Dordrecht.

Hahn, A., P. Bonfante, K. Horn, F. Pausch, and B. Hock. 1993. Production of monoclonal antibodies against surface antigens of spores from arbuscular mycorrhizal fungi by an improved immunization and screening procedure. Mycorrhiza 4:69-78.

Harrison, M.J. 2002. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50:361-389.

Hart, M.M., and R.J. Reader. 2002. Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol. 153:335-344.

Hart, M.M., and J.N. Klironomos. 2002. Diversity of arbuscular mycorrhizal fungi and ecosystem functioning. p. 225-242. *In* M.G.A. van der Heijden, and I.R. Sanders (ed.) Mycorrhial ecology. Springer, Berlin.

Hartnett, D.C., and G.W.T. Wilson. 1999. Mycorrhizae influence plant community structure and diversity in tallgrass prairie. Ecology 80:1187-1195.

Helgason, T., T.J. Daniell, R. Husband, A.H. Fitter, and J.P.W. Young. 1998. Ploughing up the wood-wide web? Nature (London) 394:431.

Helgason, T., A.H. Fitter, and J.P.W. Young. 1999. Molecular diversity of arbuscular mycorrhizal fungi colonizing *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. Mol. Ecol. 8:659-666.

Hepper, C.M., C. Azcon-Aguilar, S. Rosendahl, and R. Sen. 1988. Competition between three species of *Glomus* used as spatially separated introduced and indigenous mycorrhizal inocula for leek (*Allium porrum* L.). New Phytol. 110:207-215.

Hetrick, B.A.D., D.G. Kitt, and G.T. Wilson. 1988. Mycorrhizal dependency and growth habit of warm-season and cool-season tallgrass prairie plants. Can. J. Bot. 66:1376-1380.

Hetrick, B.A.D., D.C. Hartnett, G.W.T. Wilson, and D.J. Gibson. 1994. Effects of mycorrhizae, phosphorous availability, and plant density on yield relationships among competing tallgrass prairie grasses. Can. J. Bot. 72:168-177.

Hibbett, D.S. 2002. When good relationships go bad. Nature (London) 419:345-346.

Hijri, M., M. Hosny, D. van Tuinen, and H. Dulieu. 1999. Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zycomycota) is structured within mulinucleate spores. Fungal Genet. Biol. 26:141-151.

Hodge, A., C.D. Campbell, and A.H. Fitter. 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature (London) 413:297-299.

Hodge, A. 2001. Arbuscular mycorrhizal fungi influence decomposition of, but not plant nutrient capture from, glycine in soil. New Phytol. 151:725-734.

Hosny, M., V. Gianinazzi-Pearson, and H. Dulieu. 1998. Nuclear DNA content of eleven fungal species. Genome 41:422-428.

Hosny, M., M. Hijri, E. Passerieux, and H. Dulieu. 1999. rDNA units are highly polymorphic in *Scutellospora castanea* (Glomales, Zygomycetes). Gene 226:61-71.

Jacquot, E., D. van Tuinen, S. Gianinazzi, and V. Gianinazzi-Pearson. 2000. Monitoring species of arbuscular mycorrhizal fungi *in planta* and in soil by nested PCR: application to the study of the impact of sewage sludge. Plant Soil 226:179-188.

Jakobsen, I., L.K. Abbott, and A.D. Robson. 1992a. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L.: 1. Spread of hyphae and phosphorus inflow into roots. New Phytol. 120:371-380.

Jakobsen, I., L.K. Abbott, and A.D. Robson. 1992b. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L.: 2. Hyphal transport of ^{32}P over definded distances. New Phytol. 120:509-516.

Jansa, J., A. Mozafar, S. Banke, A. McDonald, and E. Frossard. 2002. Intra- and intersporal diversity of ITS rDNA sequences in *Glomus intraradices* assessed by cloning and sequenceing, and by SSCP analysis. Mycol. Res. 106:670-681.

Johnson, D., J.R. Leake, and D.J. Read. 2001. Novel in-growth core system enables functional studies of grassland mycorrhizal mycelial networks. New Phytol. 152:555-562.

Johnson, N.C., P.J. Copeland, R.K. Crookston, and F.L. Pfleger. 1992. Mycorrhizae: possible explanations for yield decline with continuous corn and soybean. Agron. J. 84:387-390.

Johnson, N.C., J.H. Graham, and F.A. Smith. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol. 135:575-585.

Khalil, S., T.E. Loynachan, and M.A. Tabatabai. 1994. Mycorrhizal dependency and nutrient uptake by improved and unimproved corn and soybean cultivars. Agron. J. 86:949-958.

Khalil, S., T.E. Loynachan, and M.A. Tabatabai. 1999. Plant determinants of mycorrhizal dependency in soybean. Agron. J. 91:135-141.

Kjøller, R., and S. Rosendahl. 2000. Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). Plant Soil 226:189-196.

Kjøller, R., and S. Rosendahl. 2001. Molecular diversity of glomalean (arbuscular mycorrhizal) fungi determined as distinct *Glomus* specific DNA sequences from roots of field grown peas. Mycol. Res. 105:1027-1032.

Klironomos, J.K., J. McCune, M. Hart, and J. Neville. 2000. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecol. Lett. 3:137-141.

Klironomos, J.K., and M.M. Hart. 2002. Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza 12:181-184.

Koide, R.T., and M. Li. 1991. Mycorrhizal fungi and the nutrient ecology of three oldfield annual plant species. Oecologia (Berlin) 85:403-412.

Koide, R.T. 1995. The nature of growth depressions in sunflower caused by vesicular-arbuscular mycorrhizal infection. New Phytol. 99:449-462.

Koide, R.T. 2000. Functional complementarity in the arbuscular mycorrhizal symbiosis. New Phytol. 147:233-235.

Koide, R.T., and I.A. Dickie. 2002. Effects of mycorrhizal fungi on plant populations. Plant Soil 244:307-317.

Kothari, S.K., H. Marschner, and V. Römheld. 1991. Effect of a vesicular-arbuscular mycorrhizal fungus and rhizosphere microorganisms on manganese reduction in the rhizosphere and manganese concentration in maize (*Zea mays* L.). New Phytol. 117:649-655.

Kowalchuk, G.A., S. Gerards, and J.W. Woldendorp. 1997. Detection and characterization of fungal infections of *Ammophila arenaria* (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. Appl. Environ. Microbiol. 63 :3858-3865.

Krishnaraj, P.U., and M.N. Sreenivasa. 1992. Increased root colonization by bacteria due to inoculation of vesicular-arbuscular mycorrhizal fungus in chilli (*Capsicum annuum*). Zentralbl. Mikrobiol. 147:131-133.

Lanfranco, L., P. Wyss, C. Marzachi, and P. Bonfante. 1995. Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. Mol. Ecol. 4:61-68.

Linderman, R.G. 1988. Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizoaphere effect. Phytopath. 78:366-371.

Linderman, R.G. 2000. Effects of mycorrhizas on plant tolerance to diseases. p. 345-365. *In* Y.Kapulnic, and D.D.Douds Jr. (ed.) Arbuscular Mycorrhizas: Physiology and Function. Kluwer Academic Publishers, Dordrecht.

Liu, W.T., T.L. Marsh, H. Cheng, and L. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516-4522.

Lloyd-MacGilp, S.A., S.M. Chambers, J.C. Dodd, A.H. Fitter, C.Walker, and J.P.W.Young. 1996. Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol. 133:103-111.

Lopez-Aguillon, R., and B. Mosse. 1987. Experiments on competitiveness of three endomycorrhizal fungi. Plant Soil 97:155-170.

Madan, R., C. Pankhurst, B. Hawke, and S. Smith. 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. Soil Biol. Biochem. 34:125-128.

Marschner, H., V. Römheld, and I. Cakmak. 1987. Root-induced changes of nutrient availability in the rhizosphere. J. Plant Nutr. 10:1175-1184.

Marschner, P., and D.E. Crowley. 1996. Physiological activity of a bioluminescent *Pseudomonas fluorescens* (strain 2-79) in the rhizosphere of mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L.). Soil Biol. Biochem. 28:869-876.

Marschner, P., D.E. Crowley, and R. Lieberei. 2001. Arbuscular mycorrhizal infection changes the bacterial 16 S rDNA community composition in the rhizosphere of maize. Mycorrhiza 11:297-302.

McGonigle, T.P., and A.H. Fitter. 1990. Ecological specificity of vesicular-arbuscular mycorrhizal associations. Mycol. Res. 94:120-122.

Meyer, I.R., and R.G. Linderman. 1986. Selective influence on populations of rhizosphere or rhizoplane bacteria and actionomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol. Biochem. 18:191-196.

Miller, P.D., W.W. Mulbry, S.L. Reynolds, and C.A.Patterson. 1998. A taxon-specific oligonucleotide probe for temprate zone soil isolates of *Glomus mosseae*. Mycorrhiza 8:19-27.

Millner, P.D., W.W. Mulbry, and S.L. Reynolds. 2001a. Taxon-specific oligonucleotide primers for detection of two ancient endomycorrhizal fungi, *Glomus occultum* and *Glomus brasiliamum*. FEMS Microbiol. Lett. 196:165-170.

Millner, P.D., W.W. Mulbry, and S.L. Reynolds. 2001b. Taxon-specific oligonucleotide primers for detection of *Glomus etunicatum*. Mycorrhiza 10:259-265.

Moesender, M.M., J.M. Arrieta, G. Muyzer, C. Winter, and G.J. Herndl. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 65:3518-3525.

Morton, J.B., and D. Redecker. 2001. Two new families of Glomales, *Archaeosporaceae* and *Paraglomaceae*, with two new genera *Archaeospora* and *Paraglomus*, based on concordant molecular and morphological characters. Mycologia 93:181-195.

Muyzer, G., E.C. de Waal, and A.G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59:695-700.

Nazar, R.N., E.J. Robb, and T. Volossiouk. 1996. Direct extraction of fungal DNA from soil. p. 1.3.6/1-1.3.6/8. *In* A.D.L. Akkermans, J.D. van Elsas, and F.J. De Bruijn (ed.) Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht.

Newsham, K.K., A.H. Fitter, and A.R. Watkinson. 1995. Arbuscular mycorrhiza protect an annual grass from root pathogenic fungi in the field. J. Ecol. 83:1000.

O'Connor, P.J., S.E. Smith, and F.A. Smith. 2002. Arbuscular mycorrhizas influence plant diversity and community structure in a semiarid herbland. New Phytol. 154:209-218.

Paulitz, T.C., and R.G. Linderman. 1989. Interactions between fluorescent pseudomonads and VA mycorrhizal fungi. New Phytol. 113:37-45.

Pearson, J.N., and I. Jakobsen. 1993a. Symbiotic exchange of carbon and phosphorus between cucmber and three arbuscular mycorrhizal fungi. New Phytol. 124:481-488.

Pearson, J.N., and I. Jakobsen. 1993b. The relative contribution of hyphae and roots to phorphorus uptake by arbuscular mycorrhizal plants, measured by dual labelling with ^{32}P and ^{33}P. New Phytol. 124:489-494.

Pearson, J.N., L.K. Abbott, and D.A. Jasper. 1993. Mediation of competition between two colonizing VA mycorrhizal fungi by the host plant. New Phytol. 123:93-98.

Pennanen, T., L. Paavolainen, and J. Hantula. 2001. Rapid PCR-based method for the direct analysis of fungal communities in complex environmental samples. Soil Biol. and Biochem. 33:697-699.

Posta, K., H. Marschner, and V. Römheld. 1994. Manganese reduction in the rhizosphere of mycorrhizal and non-mycorrhizal maize. Mycorrhiza 5:119-124.

Poulton, J.L., D. Bryla, R.T. Koide, and A.G. Stephenson. 2002. Mycorrhizal infection and high soil phosphorus improve vegetative growth and the female and male functions in tomato. New Phytol. 154:255-264.

Pringle, A., J.M. Moncalvo, and R. Vilgalya. 2000. High levels of variation in ribosomal DNA sequences within and among spores of a natural population of the arbuscular mycorrhizal fungus *Acaulospora colossica*. Mycologia 92:259-268.

Ravnskov, S., and I. Jakobsen. 1995. Functional compatibility in arbuscular mycorrhizas measured as hyphal P transport to the plant. New Phytol. 129:611-618.

Redecker, D., H. Thierfelder, C. Walker, and D. Werner. 1997. Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. Appl. Environ. Microbiol. 63:1756-1761.

Redecker, D., M. Hijri, H. Dulieu, and I.R. Sanders. 1999. Phylogenetic analysis of a dataset of fungal 5.8S rDNA sequences shows that highly divergent copies of internal transcribed spacers reported from *Scutellospora castanea* are of ascomycete origin. Fungal Genet. Biol. 28:238-244.

Redecker, D. 2000. Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73-89.

Redecker, D. 2002. Molecular identification and phylogeny of arbuscular mycorrhizal fungi. Plant Soil 244:67-73.

Requena, N., P. Jeffries, and J.M. Barea. 1996. Assessment of natural mycorrhizal potential in a desertified semiarid ecosystem. Appl. Environ. Microbiol. 62:842.

Requena, N., E. Perez-Solis, C. Azcon-Aguilar, P. Jeffries, and J.M. Barea. 2001. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl. Environ. Microbiol. 67:495.

Rodriguez, A., T. Dougall, J.C. Dodd, and J.P. Clapp. 2001. The large subunit ribosomal RNA genes of *Entrophospora infrequens* comprise sequences related to two different glomalean families. New Phytol. 152:159-167.

Rosendahl, S., and W. Taylor. 1997. Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. Mol. Ecol. 6:821-829.

Sainz, M.J., A. Vilariño, and J. Arisones. 1989. Competition between *Glomus tenue* and some coarse fungi for colonizing red clover roots in acid soils. Agric. Ecosyst. Environ. 29:337-340.

Sanchez-Diaz, M., and M. Honrubia. 1994. Water relations and alleviation of drought stress in mycorrhizal plants. p. 167-178. *In* S. Gianinazzi, and H. Schuepp (ed.) Impact of arbusuclar mycorrhiza on sustainable agriculture and natural ecosystems. Birkhauser, Basel.

Sanders, I.R., and R.T. Koide. 1994. Nutrient acquisition and community structure in co-occuring mycotrophic and non-mycotrophic old-field annuals. Functional Ecol. 8:77-84.

Sanders, I.R., M. Alt, K. Groppe, T. Boller, and A. Wiemken. 1995. Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol. 130:419-427.

Sanders, I.R., R.T. Koide, and D.L. Shumway. 1998. Diversity and structure in natural communities: the role of the mycorrhizal symbiosis. p. 571-593. *In* A.Varma, and B.Hock (ed.) Mycorrhiza: structure, function, molecular biology and biotechnology. Springer, Belrin.

Sanders, I.R. 1999. No sex please, we are fungi. Nature (London) 399:737-739.

Schönbeck, F. 1979. Endomycorrhiza in relation to plant diseases. p. 271-280. *In* B.Schippers, and W.Gams (ed.) Soil borne plant pathogens. Academic Press, London.

Schüßler, A. 1999. Glomales SSU rRNA gene diversity. New Phytol. 144:205-207.

Schwarzott, D., and A. Schüßler. 2001. A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AM fungal spores. Mycorrhiza 10:203-207.

Schwarzott, D., C. Walker, and A. Schüßler. 2001. *Glomus*, the largest genus of the arbuscular mycorrhizal fungi (Glomales), is nonmonophyletic. Mol. Phylogenet. Evol. 21:190-197.

Secilia, J., and D.J. Bagyaraj. 1987. Bacteria and actinomycetes associated with pot cultures of vesicular-arbuscular mycorrhizas. Can. J. Microbiol. 33:1069-1073.

Shiomi, Y., M. Nishiyama, T. Onizuka, and T. Marumoto. 1999. Comparison of bacterial community structures in the rhizosplane of tomato plants grown in soils suppressive and conductive towards bacterial wilt. Appl. Environ. Microbiol. 65:3996-4001.

Simon, L., M. Lalonde, and T.D. Bruns. 1992. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. Appl. Environ. Microbiol. 58:291-295.

Simon, L., R.C. Levesque, and M. Lalonde. 1993. Identification of endomycorrhizal fungi colonizing roots by fluorescent single-strand conformation polymorphism polymerase chain reaction. Appl. Environ. Microbiol. 59:4211-4215.

Smith, F.A., I. Jakobsen, and S.E. Smith. 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol. 147:357-366.

Smith, S., and D. Read. 1997. Mycorrhizal symbiosis. Academic Press, London.

Streitwolf-Engel, R., T. Boller, A. Wiemken, and I.R. Sanders. 1997. Clonal growth traits of two *Prunella* species are determined by co-occuring arbuscular mycorrhizal fungi from a calcareous grassland. J. Ecol. 85:181-191.

Sylvia, D.M., and D.O. Chellemi. 2001. Interactions among root-inhabiting fungi and their implications for biocontrol of root pathogens. Adv. Agron. 73:1-33.

Tadano, T., K. Ozawa, H. Sakai, M. Osaki, and H. Matsui. 1993. Secretion of acid phosphatase by the roots of crop plants under phosphorus-deficient conditions and some properties of the enzyme secreted by lupin roots. Plant Soil 155/156:95-98.

Thingstrup, I., and S. Rosendahl. 1994. Quantification of fungal activity in arbuscular mycorrhizal symbiosis by polyacrylamide gel electrophoresis and densitometry of malate dehydrogenase. Soil Biol. Biochem. 26:1483-1489.

Thomson, B.D., A.D. Robson, and L.K. Abbott. 1986. Effects of phosphorus on the formation of mycorrhizas by *Gigaspora calospora* and *Glomus fasciculatum* in relation to root carbohydrates. New Phytol. 103:751-765.

Thomson, B.D., A.D. Robson, and L.K. Abbott. 1990. Mycorrhizas formed by *Gigaspora calospora* and *Glomus fasciculatum* on subterranean clover in relation to carbohydrate concentration in roots. New Phytol. 114:217-225.

Tilman, D., C.L. Lehman, and K.T. Thomson. 1997. Plant diversity and ecosystem productivity: theoretical considerations. Proc. Natl. Acad. Sci. USA. 94:1857-1861.

Troeh, Z., and T.E. Loynachan. 2003. Endomycorrhizal fungal survival in continuous corn, soybean, and fallow. Agron. J. 95:224-230.

Turnau, K., P. Ryszka, V. Gianinazzi-Pearson, and D. van Tuinen. 2001. Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland. Mycorrhiza 10:169-174.

van der Heijden, M.G.A., T. Boller, A. Wiemken, and I.R. Sanders. 1998a. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082-2091.

van der Heijden, M.G.A., J.K. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I.R. Sanders. 1998b. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature (London) 396:69-72.

van der Heijden, M.G.A., A. Wiemken, and I.R. Sanders. 2002. Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occuring plant. New Phytol. 157:569-578.

van der Heijden, M.G.A. 2002. Arbuscular mycorrhizal fungi as a determinant of plant diversity: in search of underlying mechanisms and general principles. p. 243-265. *In* M.G.A.van der Heijden, and I.R.Sanders (ed.) Mycorrhizal ecology. Springer, Berlin.

van Elsas, J.D., and K. Smalla. 1996. Extraction of microbial community DNA from soils. p. 1.3.3/1-1.3.3/10. *In* A.D.L. Akkermans, J.D. van Elsas, and F.J. De Bruijn (ed.) Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht.

van Ray, B., and A. van Diest. 1979. Utilization of phosphate from different sources by six plant species. Plant Soil 51:577-589.

van Tuinen, D., E. Jacquot, B. Zhao, A. Gollotte, and V.Gianinazzi-Pearson. 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7:879-887.

Vandenkoornhuyse, P., S.L. Baldauf, C. Leyval, J. Straczek, and J.P.W. Young. 2002a. Extensive fungal diversity in plant roots. Science 295:2051.

Vandenkoornhuyse, P., R. Husband, T.J. Daniell, I.J. Watson, J.M. Duck, A.H. Fitter, and J.P.W. Young. 2002b. Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. FEMS Microbial Ecol. 11:1555-1564.

Waschkies, C., A. Schropp, and H. Marschner. 1994. Relations between grapevine replant disease and root colonization of grapevine (*Vitis* sp.) by fluorescent pseudomonads and endomycorrhizal fungi. Plant Soil 162:219-227.

Wilson, J.M., and M.J. Trinick. 1983. Infection development and interactions between vesicular-arbuscular mycorrhizal fungi. New Phytol. 93:543-553.

Wilson, J.M. 1984. Competition for infection between vesicular-arbuscular mycorrhizal fungi. New Phytol. 97:427-435.

Wyss, P., and P. Bonfante. 1993. Amplification of genomic DNA of arbuscular-mycorrhizal (AM) fungi by PCR using short arbitrary primers. Mycol. Res. 95:1351-1357.

Yao, M.K., R.J. Tweddell, and H. Désilets. 2002. Effects of two vesicular-arbuscular mycorrhizal fungi on the growth of micropropagated potato plantlets and on the extent of disease caused by *Rhizoctonia solani*. Mycorrhiza 12:235-242.

CHAPTER 2. RAPID, RELIABLE, AND INEXPENSIVE DNA EXTRACTION TECHNIQUES FROM TRYPAN BLUE-STAINED MYCORRHIZAL ROOTS

A paper prepared in the style for publication in Agronomy Journal

Satoshi Ishii and Thomas E. Loynachan

Abstract

Improved DNA extraction techniques from trypan blue-stained root fragments were developed for rapid, reliable, and inexpensive analyses. One cm of trypan blue-stained mycorrohizal root fragments were individually isolated, crushed by bead-beating, and purified with Chelex-100 (BioRad Laboratories, Hercules, CA). DNA extraction was also carried out using an UltraClean™ DNA isolation kit (MoBio Laboratories, Solana Beach, CA). DNA was extracted from the mycorrhizal roots of four plant species, quantified by UV absorbance, and PCR-amplified with primers specific to arbuscular mycorrhizal fungi. Results from the two extraction methods were compared. Although PCR inhibitors might still exist when using Chelex-100, appropriate dilution and employment of nested-PCR overcame this problem. Use of UltraClean™ DNA isolation kit successfully removed PCR inhibitors, but sometimes, depending on the mycorrhizal colonization within the root fragments, it also required nested-PCR to obtain bands strong enough for restriction fragment

length polymorphism (RFLP) or some other applications. In conclusion, both methods enabled us to handle hundreds of samples in a short time. UltraClean™ DNA isolation kit provided greater reliability and use of Chelex-100 provided better cost performance. Both techniques are useful for PCR-based applications to identify species and estimate species composition after measuring mycorrhizal colonization rate with trypan blue staining.

Introduction

Arbuscular mycorrhizal (AM) fungi provide their host plants with nutrients, especially the nutrients with poor mobility such as P and Zn, and in return, they receive carbohydrate for their energy source (Smith and Read, 1997). AM fungi associate with various plant species, and they positively or sometimes negatively influence their host plants (Johnson et al., 1997). AM fungi have also reported to improve plant water relations in a semiarid ecosystem (Sanchez-Diaz and Honrubia, 1994; Requena et al., 1996, 2001). Therefore, AM fungi have a strong influence on plant productivity. In natural ecosystems, diverse AM fungal species coexist in plants and soil. Efficiency of AM fungi in nutrient acquisition is different among species; some species can provide more P, while others do not promote but even reduce plant growth (Smith et al., 2000). Therefore, species composition of AM fungi in plants and soil might have important consequences on plant productivity (van der Heijden et al., 1998a, b, 2002).

The effects of AM fungi have been studied along with their colonization rates in plant roots. Conventionally, AM fungal colonization rates are measured microscopy. The most

common method is the Grid-line intersect method (Giovanetti and Mosse, 1980), in which mycorrhizal roots are stained with trypan blue. This method, however, does not provide species information because morphological characteristics of some AM fungi in roots are similar (van Tuinen et al., 1998). To overcome this problem, several methods have been applied to detect AM fungi *in planta*. Antibodies (Hahn et al., 1993), isozyme patterns (Hepper et al., 1988; Thingstrup and Rosendahl, 1994), lipid profiles (Bentivenga and Morton, 1994; Madan et al., 2002), and molecular techniques based on polymerase chain reaction (PCR) have been tested. Hepper et al. (1988) used isozyme patterns to detect AM fungi in plant roots. Although use of isozyme patterns enables us to detect metabolically active fungi, its detection sensitivity is different among fungal species (Hepper et al., 1988). Lipid profiles have potential as a biomass indicator for AM fungi but are not specific at the species level (Madan et al., 2002). PCR-based molecular techniques are the most sensitive method to identify AM fungi at the species level, but there are several problems. Among these, DNA can be amplified from metabolically inactive AM fungi such as dormant or dead spores and dead mycorrhizal roots. Also, the amount of PCR products does not necessarily reflect species biomass. Quantitative PCR can estimate the amount of template DNA and thus has a potential to estimate species biomass (Edwards et al., 1997), but species-specific primers are necessary for this purpose and, therefore, it is not adequate for a complex AM fungal community study. Terminal restriction fragment length polymorphism (T-RFLP) is a semi-quantitative DNA fingerprinting technique (Liu et al., 1997) and can be useful to study AM fungal diversity. The relative proportion of AM fungal DNA to host plant DNA derived from quantitative PCR with AM fungal specific primers and plant specific primers might alter the mycorrhizal colonization percentage, but it has not been examined yet. In the study

of plant pathology for example, Winton et al. (2002) estimated colonization of *Phaeocryptopus gaeumannii* by amplifying both pathogen and host plant DNA simultaneously and quantitatively detected each DNA with species-specific primers and TaqMan probes attached with different fluorescent dyes.

Microscopy is the best method currently to measure mycorrhizal colonization rates in roots, but it cannot identify species, while DNA-based molecular techniques are among the best for species identification. Therefore, combination of microscopy and DNA-based molecular techniques can provide both mycorrhizal colonization percentage and colonizers' species information. Further, if species from several pieces (e.g. 10 pieces) of mycorrhizal fragments per plant are identified with DNA-based molecular techniques after measuring colonization percentage, relative proportion of each AM fungus colonizing the same host can be estimated (Jacquot et al., 2000). Since this estimation requires DNA extraction and PCR-amplification of many samples, it is necessary to develop rapid and reliable AM fungal DNA extraction methods from trypan blue-stained mycorrhizal roots.

Several researchers have developed AM fungal DNA extraction techniques from mycorrhizal roots, but some of them are quite laborious because they include manual grinding (van Tuinen et al., 1998; Jacquot et al., 2000; Turnau et al., 2001), homogenization in liquid N_2 (Simon et al., 1992; Lanfranco et al., 1995; Edwards et al., 1997), razor-blade chopping (Redecker, 2000), heating (Simon et al., 1992; Di Bonito et al., 1995; Redecker, 2000), cell lyses and protein removal with enzymes and/or chemicals (Claassen et al., 1996; Lanfranco et al., 1995), and/or phenol/chloroform extraction of impurities (Simon et al., 1992; Clapp et al., 1995; Lanfranco et al., 1995; Edwards et al., 1997). Kit-based, simple, and easy DNA extraction techniques have been applied for soil (Chelius and Triprett, 1999)

and ectomycorrhial root samples (Koide and Dickie, 2002), but they are costly when large number of samples should be handled. Here, we improved upon existing DNA extraction techniques and report a rapid, reliable, and inexpensive technique to extract DNA from trypan blue-stained mycorrhizal roots.

Materials and Methods

Soil preparation

Soil was collected from the railroad site across the road west of the buildings (NW ¼, SE ¼, NE ¼, Section 9, T83W, R25W of the 5th Principal Meridian) at the Agronomy and Agricultural Engineering Research Farm, Iowa State University, located in Boone County, IA. The soil in this site is classified as Webster, which is fine-loamy, mixed, mesic Typic Endoaquolls, and a poorly drained soil. The soil contained 41% clay, 32% silt, and 27% sand; with a pH of 6.9; 54 g kg^{-1}organic matter; 38 mg kg^{-1} Bray 1 extractable P; and 136 mg kg^{-1} 1 *M* NH_4OAc extractable K. The soil was passed through a 2-mm mesh sieve and autoclaved twice with one-day duration between autoclavings and mixed with autoclaved silica sand (60:40, soil:sand by weight).

Plant culture

Four plant species were used for mycorrhizal host: alfalfa (*Medicago sativa* L. subsp. sativa), corn (*Zea mays* L.), soybean (*Glycine max* [L.] Merr.), and sudangrass (*Sorghum sudanense* [Piper] Staph). The soybean cv. BSR201 was obtained from the Iowa Crop Improvement Association, Ames, IA. Plant seeds were surface disinfected by soaking in 70% ethanol for 5 min, and rinsed with sterilized water five times. Two seeds of soybean, three seeds of corn, 20 seeds of sudan grass, or 30 seeds of alfalfa were planted in 2 kg soil: sand mixture and grown for 4 wk in the greenhouse. The growth conditions were 30°C in daytime (15 h) with natural sunlight supplemented with artificial light and 25 °C at night (9 h). The light intensity at the bench surface was 960 μmol photon m^{-2} s^{-1}. The plants were watered daily with sterile distilled water. Matric potential in soil was maintained between *ca.* 7.5 and 30 -kPa based on a soil moisture characteristic curve (Ozbek, 1998). Three wk after planting, sterile nutrient solution (Broughton and Dilworth, 1971) was applied instead of sterile distilled water. The sterile nutrient solution contained 1 m*M* $CaCl_2$, 0.1 m*M* KH_2PO_4, 10 μ*M* $FeC_6H_5O_7$, 0.25 m*M* $MgSO_4$, 0.25 m*M* K_2SO_4, 1 μ*M* $MnSO_4$, 2 μ*M* H_3BO_3, 0.5 μ*M* $ZnSO_4$, 0.2 μ*M* $CuSO_4$, 0.1 μ*M* $CoSO_4$, 0.1 μ*M* Na_2MoO_2, and 5 m*M* KNO_3. The pH of this solution was adjusted to 6.6-6.8 with 1 *M* NaOH. The P level was 1/5 of the recommended concentration proposed by Broughton and Dilworth (1971) to stimulate mycorrhizal colonization.

DNA extraction from stained mycorrhizal roots

Plant roots were harvested after 4-wk growth in the greenhouse. They were gently washed in distilled water to remove soil particles and stained with 0.05% (w/v) trypan blue in

lactoglycerol following the protocol of Brundrett et al. (1996) with minor modification. Briefly, roots of each soybean seedling were placed in a 50-mL flask and bleached with 10% (w/v) KOH at 90°C for 90 min. They were rinsed with sterile distilled water three times, and stained with 0.05% (w/v) trypan blue in lactoglycerol (lactic acid: glycerol: H_2O =1: 1: 1 by volume) at 90°C for 30 min. They were destained with sterile 50% (v/v) glycerol several times and stored at 4°C.

Genomic DNA was extracted from a one-cm fragment of trypan blue-stained mycorrhizal roots using several extraction methods. Crushing of the roots was carried out in a microcentrifuge tube by manual grinding in 40 µL of Tris buffer (100 m*M* Tris-HCl, pH 8) with micropestle (van Tuinen et al., 1998), manual grinding and powdering with liquid N_2 with micropestle, or bead beating described later. Purification of the crude DNA extract was proceeded by phenol-chloroform extraction of impurities followed by precipitation of DNA with 3 *M* sodium acetate (pH 5.2) and 2-propanol, chelation using 5% (w/v, final concentration) of Chelex-100 (BioRad Laboratories, Hercules, CA) at 90°C for 10 min (John, 1992; Di Bonito et al., 1995; van Tuinen et al., 1998), or use of silica spin column contained in UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA) (Koide and Dickie, 2002).

After intensive work, an improved DNA extraction technique from trypan blue-stained root fragments was developed for rapid, reliable, and inexpensive analyses. In this method, one-cm of trypan blue-stained mycorrohizal root fragments were individually isolated, rinsed in sterile H_2O, and placed into a microcentrifuge tube. Each root fragment was crushed by bead-beating for 10 min using approximately 50 µL (30-40 beads) of 1-mm zirconia beads (BioSpec Products, Bartlesville, OK) in 240 µL of Tris-HCl buffer (100 m*M*

Tris-HCl, pH 8.0). Bead beating was carried out for 10 min using MaxMix voltex (Fisher Scientific, Pittsburgh, PA) with adaptor that enables us to handle up to 24 samples. Crude DNA extract was incubated at 90°C for 10 min with 60 μL of 10% (w/v) Chelex-100. Chelex resin chelates heavy metals that inhibit enzymatic activity in polymerase chain reaction (PCR). Following the incubation, sample tubes were cooled on ice for approximately 1 min and centrifuged at 12 000 *g* for 5 min. 10 μL of the supernatant was diluted to 100 μL with sterile water and served as DNA template in the PCR reaction.

DNA extraction was also carried out by bead beating with silica spin column purification using UltraClean™ Microbial DNA Isolation Kit following the protocol of Koide and Dickie (2002) with some modification. Root samples were also one-cm of trypan blue-stained mycorrohizal root fragments. Each root fragment was crushed by bead-beating for 10 min using approximately 50 μL (30-40 beads) of 1-mm zirconia beads in 300 μL of bead solution (MoBio), 50 μL of M1 (detergent-based extraction solution, MoBio), and 50 μL of IRS (Inhibitor Removal Solution, MoBio). IRS is a component of UltraClean™ Soil DNA Isolation Kit (MoBio) and inactivates phenolic compounds present in soil organic matter and plant tissues that inhibit PCR reactions (Tebbe and Vahjen, 1993). The supernatant (300-350 μL) was transferred to a new tube, 100 μL of M2 (an acetate-based solution, MoBio) was added, and held at -20°C for 15 min to precipitate impurities. After the tube was centrifuged at 10 000 *g* for 1 min, 400 μL of supernatant was transferred to a new tube and 800 μL of M3 (a salt solution to help DNA with binding to the silica column, MoBio) was added and briefly mixed. 600 μL of this solution was loaded on the supplied silica spin column (MoBio), centrifuged at 10 000 *g* for 30 s, and the liquid was discarded. This step was repeated. The silica column bound with DNA was washed with 300 μL of M4

(an ethanol-based solution, MoBio) and centrifuged at 10 000 *g* for 30 s. The silica column was placed into a new tube, and DNA was eluted with 50 μL of M5 (10 m*M* Tris-HCl solution, MoBio) by centrifugation at 10 000 *g* for 30 s. This DNA solution was directly used for PCR.

The amounts of DNA extracted from several methods were calculated from the reading of UV-absorbance at 260 nm measured by a Cary 50 UV-Visible Spectrophotometer (Varian, Inc., Palo Alto, CA). The purity of DNA was also estimated by the ratio between the UV-absorbance at 260 nm and 280 nm (A_{260}/A_{280}).

PCR conditions

Nested-PCR was performed by using MiniCycler™ (MJ Research, Waltham, MA) to amplify rRNA genes including ITS region, the highly variable sequences. The universal eukaryotic primers, NS5 (White et al., 1990; 5'-AACTTAAAGGAATTGACGGAAG-3') and ITS4 (White et al., 1990; 5'-TCCTCCGCTTATTGATATGC-3'), were used in the first step, and the primer specific to *Glomus mosseae* and *G. intraradices* group, GLOM1310 primer (Redecker, 2000; 5'-AGCTAGGCTTAACATTGTTA-3') was used in combination with ITS4 primers in the second step. These primers were synthesized by Integrated DNA Technologies (Coralville, IA). The first step reaction had total volume of 7.5 μL containing 0.2 m*M* of each dNTP, 0.5 m*M* of each primer, 1.5 m*M* of $MgCl_2$, 0.2 μg μL^{-1} of Bovine Serum Albumin (BSA), 0.02 U μL^{-1} of Platinum*Taq* DNA polymerase (Invitrogen, Carlsbad, CA), the manufacturer's reaction buffer, and 1 μL of the DNA template. For negative control, 1 μL of sterile H_2O was added instead of DNA template. Platinum*Taq* polymerase accommodates hot-start PCR to minimize undesired amplification, such as primer dimmers.

PCR cycles were programmed as follows: initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 90 s, followed by final extension at 72°C for 5 min.

The amplified product of the first step PCR was diluted 1/100, and 1 μL was used as DNA template in the second step reaction containing the same reaction mixture with the total volume of 12.5 μL. The same PCR cycle program was used in the second step as well. Aliquots of 3.0 μL were run on a 1.2% (w/v) agarose gel and stained with ethidium bromide to confirm the amplification of products with the desired size (approximately 1000 bp).

Statistical analyses

All numerical data were statistically analyzed by one-way ANOVA using SAS program.

Results

DNA extraction from stained mycorrhizal roots

Table 2-1 compares DNA amount, purity, and the final concentration of DNA solution between the two root crushing methods: manual grinding and bead beating. Host plant was soybean. No major differences were observed except final concentration. Since bead beating requires six-times more solution volume than manual grinding, this method provides lower final concentration. We also manually ground samples in liquid N_2 to

completely homogenize root samples. This root crushing method provided the highest amount of DNA, but because of its laboriousness we did not use it further.

Table 2-1. Comparison between the two root crushing methods in DNA extracted from soybean roots (mean ± SEM, n=3).

Root crushing	Amount	Final conc.	Purity
	μg / sample	μg / mL	A_{260}/A_{280}
Manual grinding	1.225 ± 0.200	2.450 ± 0.401	1.323 ± 0.152
Bead beating	1.669 ± 0.356	0.557 ± 0.119	1.225 ± 0.025

Along with the root crushing method, we also tried several DNA purification techniques: phenol-chloroform extraction of impurities followed by precipitation of DNA with 3 *M* sodium acetate (pH 5.2) and 2-propanol, chelating PCR inhibitors using Chelex-100, and DNA purification with the silica spin column contained in UltraClean™ Microbial DNA Isolation Kit. Although phenol-chloroform purification somewhat improved DNA purity (data not shown), this process lost much DNA and took a lot of time and labor. Therefore, this purification method was avoided for further research.

Amount and purity of DNA extracted from trypan blue-stained mycorrhizal roots were compared between our improved DNA extraction technique using bead beating and Chelex-100 and bead beating with silica spin column purification method using UltraClean™ Microbial DNA Isolation Kit (Tables 2-2 and 2-3). Bead beating with Chelex-100 purification provided greater amount of DNA per sample (per one-cm trypan blue-stained mycorrhizal root fragment) than the use of UltraClean™ Kit. Purity of DNA (A_{260}/A_{280}),

Table 2-2 Amount of DNA extracted by the two methods: bead beating (BB) with Chelex-100 purification and use of UltraClean™ Microbial DNA Isolation Kit (mean ± SEM, n=3).

Host plant	BB /Chelex-100	UltraClean™ Kit	Level of
	μg / sample		significance
Alfalfa	1.463 ± 0.075	0.338 ± 0.038	**
Corn	1.063 ± 0.152	0.492 ± 0.033	*
Soybean	1.669 ± 0.356	0.363 ± 0.038	
Sudan grass	1.188 ± 0.237	0.425 ± 0.025	

*, ** Significantly different between BB/Chelex-100 and UltraClean™ Kit at the 0.05 and 0.01 probability levels, respectively.

Table 2-3 Purity of DNA extracted by the two methods (mean ± SEM, n=3).

Host plant	BB /Chelex-100	UltraClean™ Kit	Level of
	A_{260}/A_{280}		significance
Alfalfa	1.151 ± 0.029	1.822 ± 0.322	**
Corn	1.261 ± 0.031	1.348 ± 0.035	*
Soybean	1.225 ± 0.025	1.818 ± 0.040	**
Sudan grass	1.224 ± 0.030	1.618 ± 0.018	

*, ** Significantly different between BB/Chelex-100 and UltraClean™ Kit at the 0.05 and 0.01 probability levels, respectively.

Table 2-4 Final concentration of DNA extracted by the two methods (mean ± SEM, n=3).

Host plant	BB /Chelex-100	UltraClean™ Kit	Level of
	μg / mL		significance
Alfalfa	0.488 ± 0.025	6.750 ± 0.750	**
Corn	0.355 ± 0.051	9.883 ± 0.667	
Soybean	0.557 ± 0.119	7.250 ± 0.750	*
Sudan grass	0.396 ± 0.079	8.500 ± 0.500	***

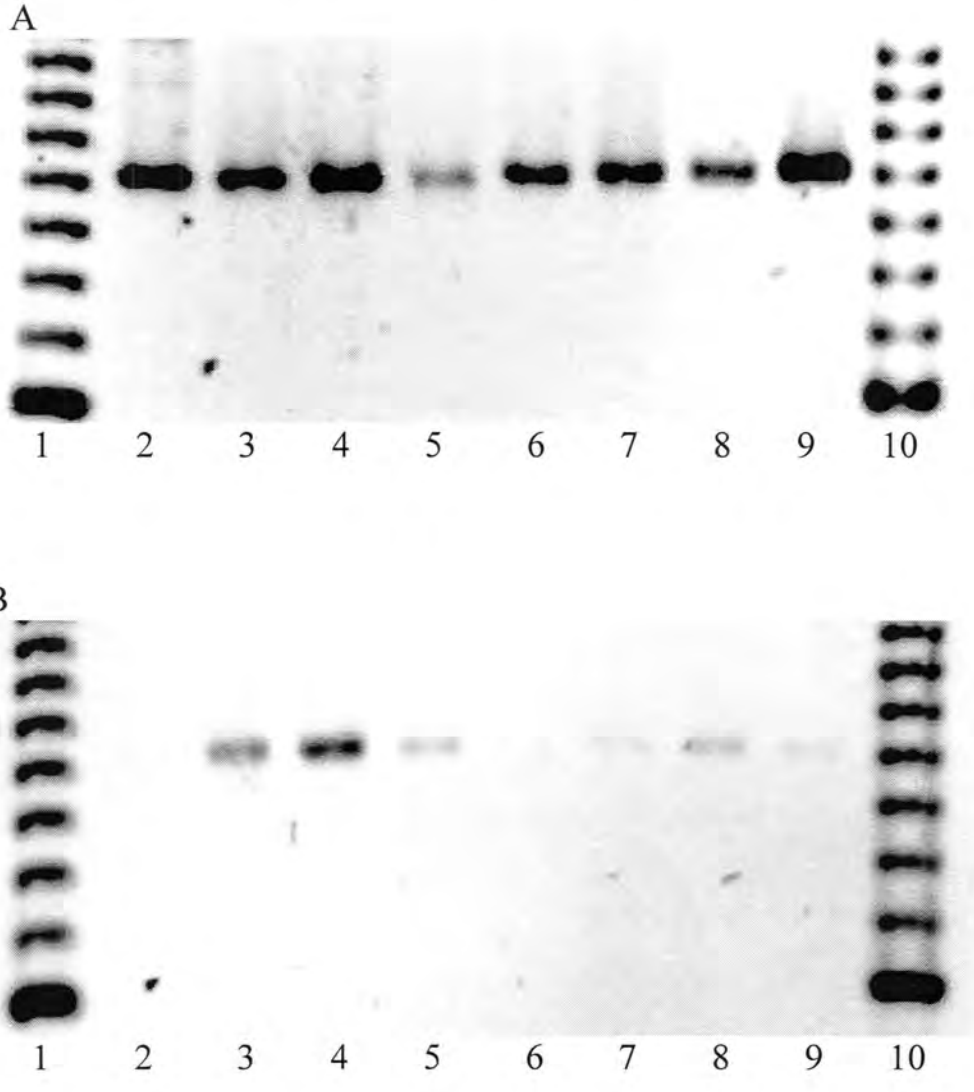
*, **, *** Significantly different between BB/Chelex-100 and UltraClean™ Kit at the 0.05, 0.01, and 0.001 probability levels, respectively.

however, was lower in bead beating with Chelex-100 purification compared to UltraClean™ Kit (pure preparation of DNA has an A_{260}/A_{280} ratio of 1.6-1.8), suggesting impurities such as proteins and phenolic compounds might exist in the extract of the method using bead beating and Chelex-100. Since proteins, RNA, and phenolic compounds also absorb UV at 260 nm, the amount of DNA shown in Table 2-2 might be overestimated, especially in bead beating with Chelex-100 purification. All these observations were consistent in the four host plants.

Although the amount of DNA in bead beating with Chelex-100 purification was greater than that in UltraClean™ Kit, the final concentration of DNA extracted by this method was lower (Table 2-4). The final volume of the method using bead beating with Chelex-100 was 300 μL and it is diluted 10-fold, while the final volume of the method using UltraClean™ Kit was 50 μL and no dilution was applied. Since 1.0 μL each was used as a template for PCR reactions, the amount of DNA in PCR reactions was *ca.* 4-8 ng per 100 μL, which is very low, in bead beating with Chelex-100 purification and *ca.* 80-140 ng per 100 μL in UltraClean™ Kit. DNA extracted from stained mycorrhizal roots would be derived mainly from plants and fungi, but also from bacteria, nematodes, and other organisms. Therefore, copy number of target DNA template for PCR reactions might be extremely low, requiring double-step PCR or nested-PCR. PCR products on 1.2% (w/v) agarose gel are shown in Fig. 2-1.

The percentage of samples successfully amplified by PCR was 71% (107/151) in bead beating with Chelex-100 purification and 94% (17/18) in bead beating with silica spin column purification. In the DNA extraction method using bead beating with Chelex-100 purification, further dilution (100-fold) of DNA solution provided successful amplification in

Fig. 2-1 PCR products amplified with *Glomus mosseae/intraradices* specific primers by nested-PCR (A) and one-step PCR (B). Lane 1 and 10, 100 bp DNA ladder (from bottom to top, 600, 700, 800, 900, 1000, 1100, 1200, and 1300 bp); lane 2-4, DNA was extracted by UltraClean™ Kit from alfalfa (lane 2), corn (lane 3), soybean (lane 4), and sudan grass (lane 5); lane 6-7, DNA was extracted by bead beating with Chelex-100 purification from alfalfa (lane 6), corn (lane 7), soybean (lane 8), and sudan grass (lane 9).



18 out of 23 attempts (78%) for DNA samples not amplified when they were diluted 10-fold. Negative controls were always clean (no amplification observed).

Discussion

We simplify the current DNA extraction protocol (van Tuinen et al., 1998) by incorporating bead beating step to root crushing. Although manual grinding provided denser final DNA concentration, bead beating was less laborious and less time consuming than manual grinding; therefore, we can process more samples in a shorter time. Since bead beating enabled us to handle up to 24 samples with the attachment to the voltex mixer and even up to 192 samples with a commercially available bead beater (e. g. Mini-BeadBeater-96™, BioSpec Product) at the same time, this method is useful especially when many samples need to be analyzed.

Purification with phenol-chloroform extraction followed by precipitation with alcohol worked well, but the procedure lost much DNA and took time and labor to process. Furthermore, phenol is a hazardous chemical. Therefore, this purification method is not adequate for samples with small amounts of DNA and for laboratory research requiring analysis of large numbers. Purification using Chelex-100 did not remove all PCR inhibitors, but appropriate dilution (10- or 100-fold) overcame the inhibition for PCR reaction in most cases. Purification using Chelex-100 is simple and requires only a short time, therefore, is appropriate when many samples need to be processed.

We also modified the DNA extraction method proposed by Koide and Dickie (2002) to be suitable for trypan blue-stained mycorrhizal roots. Their method employed bead beating with silica spin column purification using UltraClean™ Microbial DNA Isolation Kit. We used smaller beads than they proposed to crush the short and soft roots efficiently, and several steps such as root freezing and bead beating without solution were avoided to shorten the processing time. Since mycorrhizal roots were partially digested by KOH prior to trypan blue-staining and DNA extraction, the amount of PCR inhibitors such as phenolic compounds might be less than non-digested roots. Therefore, use of IRS might not be necessarily required. In our attempt, three samples were all successfully amplified by PCR from DNA extracted without IRS treatment. More data are needed to verify the necessity of IRS.

Comparison between bead beating with Chelex-100 purification and bead beating with silica spin column purification using UltraClean™ Microbial DNA Isolation Kit showed that the latter method provided purer DNA solution with denser final concentration. The former method, however, was less expensive and less time consuming. Both methods provided strong bands by nested-PCR, but percentage of successful amplification was greater in the method using UltraClean™ Microbial DNA Isolation Kit. The difference in percentage of successful amplification might be due to the different amount of PCR inhibitors in the DNA extract. The results in DNA purity (A_{260}/A_{280}) shown in Table 2-3 suggested that the method using UltraClean™ Microbial DNA Isolation Kit provided purer DNA than the method using Chelex-100. Inhibition in PCR reaction can be minimized by 10- or 100-fold dilution. *Taq* DNA polymerase is sensitive for DNA template; therefore, dilution of DNA extract does not affect DNA amplification itself. Use of bovine serum

albumin (BSA) in PCR helped the activity of *Taq* DNA polymerase and improved the PCR results (Kreader, 1996).

Final DNA concentration of both methods did not contain AM fungal DNA high enough for one-step PCR when they were extracted from one-cm length of trypan blue-stained mycorrhizal root fragment (Fig. 2-1). Longer root fragments could overcome this problem but we did not try that because we were interested in estimating the species proportion of AM fungi colonizing the same roots; shorter root fragments with more replication was better than longer root fragments with less replication for this purpose.

In conclusion, bead beating was a rapid and simple root crushing method and useful especially when we need to analyze many samples. For rapid, simple, and reliable DNA purification, we believe both silica spin column and Chelex-100 work well. Although purification with silica spin column using UltraClean™ Microbial DNA Isolation Kit provided purer DNA solution, purification with Chelex-100 and appropriate sample dilution overcame PCR inhibition in most cases. Since use of Chelex-100 is less expensive than UltraClean™ Microbial DNA Isolation Kit, this method saves experimental costs when we need to handle many samples.

Both DNA extraction methods were rapid and useful, and we could choose one based on our needs. Bead beating with silica spin column purification using UltraClean™ Microbial DNA Isolation Kit provided greater reliability and bead beating with Chelex-100 purification provided better cost performance. Both techniques were useful for PCR-based applications such as restriction fragment length polymorphism (RFLP) to identify species and estimate species composition after measuring mycorrhizal colonization rate with trypan blue staining.

References

Bentivenga, S.P., and J.B. Morton. 1994. Stability and heritability of fatty acid methyl ester profiles of glomalean endomycorrhizal fungi. Mycol. Res. 98:1419-1426.

Broughton, W.J., and M.J. Dilworth. 1971. Control of leghaemoglobin synthesis in snake beans. Biochem. J. 125:1075-1080.

Brundrett, M., N. Bougher, B. Dell, T. Grove, and N. Malajczuk. 1996. Working with mycorrhizas in forestry and agriculture. Australian Centre for International Agricultural Rresearch, Canberra.

Chelius, M.K., and E.W. Triprett. 1999. Rapid detection of arbuscular mycorrhizae in roots and soil of an intensively managed turfgrass system by PCR amplification of small subunit rDNA. Mycorrhiza 9:61-64.

Claasen, V.P., R.J. Zasoski, and B.M. Tyler. 1996. A method for direct soil extractrion and PCR amplification of endomycorrhizal fungal DNA. Mycorrhiza 6:447-450.

Clapp, J.P., J.P.W. Young, J.W. Merryweather, and A.H. Fitter. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol. 130:259-265.

Di Bonito, R., M.L. Elliott, and E.A. Des Jardin. 1995. Detection of an arbuscular mycorrhizal fungus in roots of different plant speices wih the PCR. Appl. Environ. Microbiol. 61:2809-2810.

Edwards, S.G., A.H. Fitter, and J.P.W. Young. 1997. Quantification of an arbuscular mycorrhizal fungus, *Glomus mosseae*, within plant roots by competitive polymerase chain reaction. Mycol. Res. 101:1440-1444.

Giovannetti, M., and B. Mosse. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84:489-500.

Hahn, A., P. Bonfante, K. Horn, F. Pausch, and B. Hock. 1993. Production of monoclonal antibodies against surface antigens of spores from arbuscular mycorrhizal fungi by an improved immunization and screening procedure. Mycorrhiza 4:69-78.

Hepper, C.M., C. Azcon-Aguilar, S. Rosendahl, and R. Sen. 1988. Competition between three species of *Glomus* used as spatially separated introduced and indigenous mycorrhizal inocula for leek (*Allium porrum* L.). New Phytol. 110:207-215.

Jacquot, E., D. van Tuinen, S. Gianinazzi, and V. Gianinazzi-Pearson. 2000. Monitoring species of arbuscular mycorrhizal fungi *in planta* and in soil by nested PCR: application to the study of the impact of sewage sludge. Plant Soil 226:179-188.

John, M.E. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucleic Acids Res. 20:2381.

Johnson, N.C., J.H. Graham, and F.A. Smith. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol. 135:575-585.

Koide, R.T., and I.A. Dickie. 2002. Kit-based, low-toxicity method for extracting and purifying fungal DNA from ectomycorrhizal roots. BioTech. 32:52-56.

Kreader, C.A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Appl. Environ. Microbiol. 62:1102-1106.

Liu, W.-T., T.L. Marsh, H. Cheng, and L. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516-4522.

Madan, R., C. Pankhurst, B. Hawke, and S. Smith. 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. Soil Biol. Biochem. 34:125-128.

Ozbek, M. 1998. Effects of soil pH and soil water potential on arbuscular-mycorrhizal fungal colonization of soybean. M.S. thesis, Iowa State University, Ames, IA

Redecker, D. 2000. Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73-89.

Requena, N., P. Jeffries, and J.M. Barea. 1996. Assessment of natural mycorrhizal potential in a desertified semiarid ecosystem. Appl. Environ. Microbiol. 62:842.

Requena, N., E. Perez-Solis, C. Azcon-Aguilar, P. Jeffries, and J.M. Barea. 2001. Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl. Environ. Microbiol. 67:495.

Sanchez-Diaz, M., and M. Honrubia. 1994. Water relations and alleviation of drought stress in mycorrhizal plants. p. 167-178. *In* S. Gianinazzi, and H. Schuepp (ed.) Impact of arbusuclar mycorrhiza on sustainable agriculture and natural ecosystems. Birkhauser, Basel.

Smith, F.A., I. Jakobsen, and S.E. Smith. 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol. 147:357-366.

Smith, S., and D. Read. 1997. Mycorrhizal symbiosis. Academic Press, London.

Tebbe, C.C., and W. Vahjen. 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and yeast. Appl. Environ. Microbiol. 59:2657-2665.

Thingstrup, I., and S. Rosendahl. 1994. Quantification of fungal activity in arbuscular mycorrhizal symbiosis by polyacrylamide gel electrophoresis and densitometry of malate dehydrogenase. Soil Biol. Biochem. 26:1483-1489.

Turnau, K., P. Ryszka, V. Gianinazzi-Pearson, and D. van Tuinen. 2001. Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland. Mycorrhiza 10:169-174.

van der Heijden, M.G.A., J.K. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I.R. Sanders. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature (London) 396:69-72.

van der Heijden, M.G.A., T. Boller, A. Wiemken, and I.R. Sanders. 1998. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082-2091.

van der Heijden, M.G.A., A. Wiemken, and I.R. Sanders. 2002. Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occuring plant. New Phytol. 157:569-578.

van Tuinen,D., E.Jacquot, B.Zhao, A.Gollotte, and V.Gianinazzi-Pearson. 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7:879-887.

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315-322. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (ed.) PCR protocols, a guide to methods and applications. Academic Press, London.

Winton, L.M., J.K. Stone, L.S. Watrud, and E.M. Hansen. 2002. Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopath. 92:112-116.

Appendix

Table A. Raw data for Table 2-1. Comparison between the two root crushing methods in DNA extracted from soybean roots.

Root crushing	Absorbance		Amount	Purity	Final conc.
	A_{260}	A_{280}	µg / sample	A_{260}/A_{280}	µg / mL
Manual grinding	0.042	0.035	1.050	1.200	2.100
	0.065	0.040	1.625	1.625	3.250
	0.008	0.007	1.000	1.143	2.000
Bead beating	0.054	0.045	2.025	1.200	0.675
	0.035	0.028	1.313	1.250	0.438
	ND†	ND†	ND†	ND†	ND†

†ND, not determined.

Table B. Raw data for Table 2-2, 2-3, and 2-4. Amount, purity, and final concentration of DNA extracted by the two methods, bead beating (BB) with Chelex-100 purification and use of UltraClean™ Microbial DNA Isolation Kit.

Host plant	Root crushing	Absorbance		Amount	Purity	Final conc.
		A_{260}	A_{280}	µg / sample	A_{260}/A_{280}	µg / mL
Alfalfa	BB /Chelex-100	0.035	0.029	1.313	1.207	0.438
		0.041	0.036	1.538	1.139	0.513
		0.041	0.037	1.538	1.108	0.513
	UltraClean™ Kit	0.015	0.007	0.375	2.143	7.500
		0.012	0.008	0.300	1.500	6.000
		0.029	0.023	0.725	1.261	14.50
Corn	BB /Chelex-100	0.029	0.023	1.088	1.262	0.363
		0.021	0.016	0.788	1.313	0.263
		0.035	0.029	1.313	1.207	0.438
	UltraClean™ Kit	0.021	0.016	0.525	1.313	10.50
		0.021	0.016	0.525	1.313	10.50
		0.017	0.012	0.425	1.417	8.500
Soybean	BB /Chelex-100	0.054	0.045	2.025	1.200	0.675
		0.035	0.028	1.313	1.250	0.438
		ND†	ND†	ND†	ND†	ND†
	UltraClean™ Kit	0.016	0.009	0.400	1.778	8.000
		0.013	0.007	0.325	1.857	6.500
		ND†	ND†	ND†	ND†	ND†
Sudan grass	BB /Chelex-100	0.023	0.018	0.863	1.278	0.288
		0.044	0.036	1.650	1.222	0.550
		0.028	0.022	1.050	1.173	0.350
	UltraClean™ Kit	0.018	0.011	0.450	1.636	9.000
		0.016	0.010	0.400	1.600	8.000
		0.045	0.036	1.125	1.250	22.50

†ND, not determined.

CHAPTER 3. DIFFERENT COLONIZATION RATES OF AN ARBUSCULAR MYCORRHIZAL FUNGUS BETWEEN TWO SOYBEAN CULTIVARS

A paper prepared in the style for publication in Agronomy Journal

Satoshi Ishii and Thomas E. Loynachan

Abstract

Colonization rates of an arbuscular mycorrhizal fungus, *Glomus mosseae*, within two soybean cultivars, BSR201 and Mandarin, were studied along with plant growth. Although *G. mosseae* did not promote plant growth significantly in this study, mycorrhizal colonization rates were different between the two soybean varieties at both 4 and 8 wk after planting. No relationship was observed between mycorrhizal colonization rate and plant growth. Differences in mycorrhizal colonization rates might be due to different amounts of carbohydrate supplied from the host plants and/or different activities in plant responses to the infection of *G. mosseae*. When both BSR201 and Mandarin were planted in the same pot, colonization rates of Mandarin were increased, probably due to the activity of BSR201 and its mycorrhizal roots.

Introduction

Arbuscular mycorrhizal (AM) fungi colonize within the roots of more than 80% of terrestrial plants (Smith and Read, 1997). Generally, AM fungi provide nutrients and water to their host plants, and in return, they receive carbohydrate as their energy source (Smith and Read, 1997). AM fungi also contribute to maintain plant biodiversity (Streitwolf-Engel et al., 1997; van der Heijden et al., 1998a, b, 2002; Klironomus et al., 2000) probably because plants can better utilize limiting nutrient resources with AM fungi, resulting in more ecosystem productivity and richness in small plant species (Klironomus et al., 2000).

Conversely, plants also influence AM fungal community structure probably because of their preferences on the mycorrhizal partner (van der Heijden et al., 1998b). AM fungal colonization rates are different among host plants (Smith and Read, 1997), suggesting plants have different responses to AM fungi. Mycorrhizal dependency also varies among plant species (Hetrick et al., 1988; Koide and Li, 1991) and even among varieties (Khalil et al., 1994 & 1999).

Although different effects of the plant on AM fungi have been reported, impact of plant diversity on mycorrhizal colonization has not been studied well. In this study, we investigated the impact of host plants on AM fungal colonization using two soybean varieties. Also, effects of mixed planting of the two soybean varieties on mycorrhizal colonization and soybean growth were examined. This study could help us to understand the interaction among plants and impact on AM fungi.

Materials and Methods

Soil preparation

The same soil was used as previously described. Soil preparation and characteristics of the soil sample were described in Chapter 2.

Mycorrhizal inoculum

The AM fungus, *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (isolate BEG 83 same as DN990), was obtained from the International Culture Collection of Arbuscular and VA Mycorrhizal Fungi (INVAM), Morgantown, WV. Initial inoculum was propagated with soybean (*Glycine max* [L.] Merr.) cv. Mandarin for 10 wk in the greenhouse. The growth conditions were described in Chapter 2. Soil containing AM fungal hyphae, mycorrhizal roots, and spores was used as an inoculum.

Plant culture

Two soybean cultivars, BSR201 and Mandarin, were obtained from the Iowa Crop Improvement Association, Ames, IA. BSR201 is a determinate and improved cultivar that has resistance against brown stem rot (BSR) disease caused by the soil-borne fungus, *Phialophora gregata* (Allington & Chamberlain) Gams. Mandarin is an indeterminate and unimproved cultivar. Determinate varieties start flowering when they nearly reached to their final height, and their flowering occurs about the same time in the top and the bottom of the plant (Fehr and Caviness, 1977). Indeterminate varieties, however, start their flowering when they achieved less than half of their final height (Fehr and Caviness, 1977).

Both BSR201 and Mandarin were also used in the mycorrhizal study by Khalil et al. (1994). In their study, Mandarin had higher mycorrhizal dependency, while BSR201 had slightly higher mycorrhizal colonization rates.

Plant culture and the greenhouse experiment

Soybean seeds were surface disinfected by soaking in 70% ethanol for 5 min, and rinsed with sterilized water five times. Seeds were pregerminated in sterile distilled water for 2 d. Two seeds of each soybean cultivar were planted in a 15-cm pot containing 2 kg soil: sand mixture (40:60 by weight). One seed each of the two cultivars was also planted together in the same pot. They were grown for 4 and 8 wk in the greenhouse. Pots were arranged in a completely randomized design. The growth conditions were described in Chapter 2.

Plant harvesting

Four and 8 wk after planting, soybean shoots and roots were harvested. Both BSR201 and Mandarin were at early vegetative stage (V3) after 4-wk growth, but their growth stages were different after 8-wk growth. BSR201 was at early reproductive stage (R2), in which flowering was started at all nodes. Mandarin was at early-to-late reproductive stage (R5), in which seeds were beginning to form.

Shoots were oven-dried in paper bags at 105°C overnight, and their dry weight was recorded. For Mandarin cultivar, pods and seeds weights were also recorded and vegetative shoot dry weight was calculated by subtracting the pod and seed weight from the shoot dry weight.

Mycorrhizal colonization rate

Plant roots were gently washed in distilled water to remove soil particles and stained with 0.05% (w/v) trypan blue in lactoglycerol following the protocol by Brundrett et al. (1996) with minor modification, which is described briefly in Chapter 2. Stained soybean roots were cut into one-cm fragments, and mycorrhizal colonization rates were measured by the Gridline Intersect Method (Giovannetti and Mosse, 1980) under 40 × magnification using 0.25-0.30 g (fresh weight) of the root fragments.

Statistical analyses

All numerical data were statistically analyzed by one-way ANOVA using SAS program. Colonization rates among plants and among pots were not significantly different; therefore, colonization rates were analyzed based on treatments. Multi-factorial ANOVA (PROC GLM) was used to analyze the differences in shoot dry weight among the treatments. Scheffe's test was used to group treatments based on colonization rates.

Results

Shoot dry weight

Plant growth was estimated by shoot dry weight shown in Table 3-1. In Table 3-2, no significant differences in shoot dry weight were observed between inocula (*G. mosseae vs.* Control), between cultivars (BSR201 *vs.* Mandarin), between planting (single *vs.* mixed), and their interactions in both 4- and 8-wk growth. Vegetative shoot dry weight was also not

Table 3-1. Soybean shoot dry weight (g) and vegetative shoot dry weight (mean ± SEM, n=6 for single cultivar and n=3 for mixed cultivar).

	Inoculum	*Glomus mosseae*				Control			
	Cultivar	BSR201		Mandarin		BSR201		Mandarin	
	Planting	Single	Mixed	Single	Mixed	Single	Mixed	Single	Mixed
		g							
4 wk	SDW†	0.58 ± 0.061	0.44 ± 0.078	0.50 ± 0.029	0.33 ± 0.034	0.47 ± 0.086	0.54 ± 0.029	0.60 ± 0.107	0.67 ± 0.107
8 wk	SDW†	2.72 ± 0.440	2.42 ± 0.806	2.36 ± 0.205	1.81 ± 0.183	1.61 ± 0.231	1.75 ± 0.582	2.13 ± 0.297	2.04 ± 0.669
	VSDW‡			1.45 ± 0.173	1.10 ± 0.155			1.33 ± 0.182	1.37 ± 0.412

†SDW, shoot dry weight.

‡VSDW, vegetative shoot dry weight.

Table 3-2. Soybean shoot dry weight infected with *Glomus mosseae* (mean ± SEM, n = 6 for single cultivar and n = 3 for mixed cultivar).

Treatments, source of variation	DF†	Mean Square	*F* value	*P* > *F*
4 wk				
Inoculum (*G. mosseae vs.* Control)	1	0.0891	3.000	0.09
Cultivars (BSR201 *vs.* Mandarin)	1	0.0037	0.120	0.73
Planting (Single *vs.* Mixed)	1	0.0121	0.410	0.53
Inoculum x Cultivar	1	0.0968	3.260	0.08
Cultivar x Planting	1	0.0008	0.030	0.87
Inoculum x Cultivar x Planting	2	0.0503	1.690	0.20
8 wk				
Inoculum (*G. mosseae vs.* Control)	1	1.5842	2.200	0.15
Cultivars (BSR201 *vs.* Mandarin)	1	0.0139	0.020	0.89
Planting (Single *vs.* Mixed)	1	0.3121	0.430	0.52
Inoculum x Cultivar	1	1.5900	2.210	0.15
Cultivar x Planting	1	0.1120	0.160	0.70
Inoculum x Cultivar x Planting	2	0.2025	0.280	0.76

†DF, degree of freedom.

significantly different among the treatments. Although, the difference in vegetative shoot dry weight was not significant at P = 0.05 levels, BSR201 had relatively more vegetative area (more leaves and stems) 8 wk after planting, compared with Mandarin. The reason why Mandarin, an indeterminate variety, had less vegetative area than BSR201, a determinate variety, is probably because energy and nutrition were used for flowering and formation of pod and seeds in Mandarin.

Shoot dry weight of BSR201 was increased with the inoculation of *G. mosseae* compared with the control, but the differences in shoot dry weight and vegetative shoot dry weight between BSR201 and control and between Mandarin and control were not significantly different.

Mycorrhizal colonization rates

Mycorrhizal colonization rates, however, were significantly different at the P = 0.05 level between BSR201 and Mandarin after both 4 and 8 wk (Fig. 3-1 and 3-2). Based on Scheffe's test, BSR201 (both single cultivar and mixed cultivar) and Mandarin mixed cultivar were shown to have significantly different mycorrhizal colonization rates from Mandarin single cultivar. Mycorrhizal colonization rates of control pots were always 0%.

No strong relationships were observed between colonization rates and shoot dry weight (R^2 = 0.0105 at 4 wk and 0.0023 at 8 wk) and between colonization rate and vegetative shoot dry weight (R^2 = 0.2366).

Fig. 3-1. Mycorrhizal colonization rates 4 wk after planting (mean ± SEM, n = 18 for single cultivar and n = 9 for mixed cultivar).

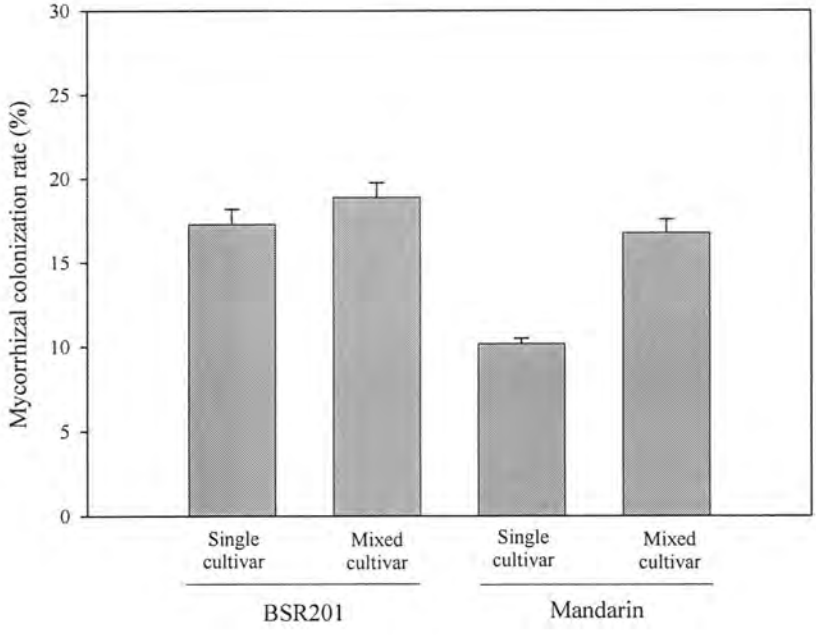
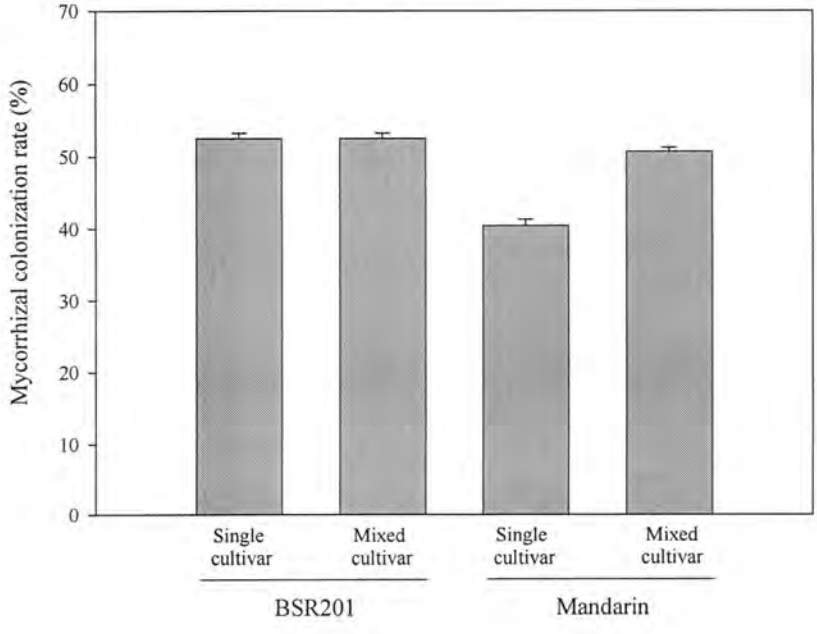


Fig. 3-2. Mycorrhizal colonization rates 8 wk after planting (mean ± SEM, n = 18 for single cultivar and n = 9 for mixed cultivar).



Discussion

Shoot dry weight

In this study, we did not observe a significant increase in shoot dry weight and vegetative shoot dry weight with the inoculation of *G. mosseae* (Tables 3-1 and 3-2). This is probably because soybean shoot dry weight had higher standard deviation, the amount of AM fungal inoculum was not enough , and/or our growth periods (4 and 8 wk) were not long enough in order to observe significant differences.

No differences were observed between single *vs.* mixed or BSR201 *vs.* Mandarin in short dry weight and vegetative shoot dry weight in inoculated treatments (Table 3-2), suggesting neither soybean cultivar nor a means of planting (single or mixed cultivar in the same plot) strongly affected soybean growth.

Mycorrhizal colonization rates

Mycorrhizal colonization rates, however, were influenced by both soybean cultivar and means of planting (Fig. 3-1 and 3-2). Both after 4- and 8-wk growth, mycorrhizal colonization rates in BSR201 were higher than those in Mandarin single cultivar.

There are several possible explanations for this observation. First, BSR201 might provide more carbohydrate than Mandarin because BSR201 had relatively more vegetative area. Larger leaf area of BSR201 compared with Mandarin was also reported by Khalil et al. (1994). No relationship was observed between mycorrhizal colonization rates and root dry weight and between mycorrhizal colonization rates and vegetative shoot dry weight, suggesting that both shoot dry weight and vegetative shoot dry weight did not explain the

difference in mycorrhizal colonization rates between BSR201 and Mandarin. Since plant carbohydrate supply to AM fungi may not necessarily reflect plant growth and leaf area, direct measurement of carbohydrate supply is necessary to verify the relationship between mycorrhizal colonization rates and plant carbohydrate supply.

Second, differences in mycorrhizal colonization between BSR201 and Mandarin at 4 wk indicated that the early plant responses such as elicitor degradation, signaling between plants and AM fungi, and/or recognition of AM fungi (Roussel et al., 2001), might vary between the two soybean varieties. More research is needed to elucidate the differences in plant response activities against mycorrhizal infection and colonization.

Information obtained in this study was not enough to conclude what caused the differences in mycorrhizal colonization between BSR201 and Mandarin. More research is necessary to clarify the reason of the difference in mycorrhizal colonization rates between BSR201 and Mandarin.

Effects of mixed inoculation

The most interesting observation in this study was that the mycorrhizal colonization rates in Mandarin were increased when BSR201 was planted in the same pot, suggesting BSR201 helped and/or enhanced Mandarin infection by *G. mosseae*. Possible explanations for this observation are that secondary infection from mycorrhizal roots of BSR201 occurred in the Mandarin roots and/or more signaling chemicals were secreted from BSR201 that activated the infection units (such as spores, hyphae, and mycorrhizal roots) of *G. mosseae*. Although the former hypothesis may explain the differences in mycorrhizal colonization rates 8 wk after planting, it seems unlikely to explain the differences after 4-wk growth

because *G. mosseae* generally infect from 10-20 d (Hart and Reader, 2002) and 4 wk are not long enough for secondary infection. In order to confirm the latter hypothesis, more study is necessary including the molecular biology of signaling pathways of mycorrhizal infection in both BSR201 and Mandarin.

Conclusion

Although differences in mycorrhizal colonization rates between BSR201 and Mandarin were observed, the factors influencing this difference are still unclear. Different amounts of carbohydrate supply from the host plants and/or different activities in plant responses to the infection of *G. mosseae* might explain this observation but more research is necessary to verify these hypotheses. In this study, no relationship was observed between mycorrhizal colonization rates and shoot dry weight.

Our research also showed that mixed planting affected the mycorrhizal colonization. It might be possible, therefore, to increase the mycorrhizal colonization rates of some plants and increase their growth by planting other mycorrhizal plants next to them. This observation might also support that the plant community influences AM fungal colonization in plant roots. The mechanism of the interaction among host plants and AM fungi should be studied further.

This study, along with Chapter 4, might become a first step to understand the complex interactions among plant community and AM fungal community in natural ecosystems.

References

Fehr, W.R., and C.E. Caviness. 1977. Stages of soybean development. Spec. Rep. 80. Iowa Agric.Home Econ. Exp. Stn., Iowa State Univ., Ames, IA.

Hetrick, B.A.D., D.G. Kitt, and G.T. Wilson. 1988. Mycorrhizal dependency and growth habit of warm-season and cool-season tallgrass prairie plants. Can. J. Bot. 66:1376-1380.

Khalil, S., T.E. Loynachan, and M.A. Tabatabai. 1994. Mycorrhizal dependency and nutrient uptake by improved and unimproved corn and soybean cultivars. Agron. J. 86:949-958.

Khalil, S., T.E. Loynachan, and M.A. Tabatabai. 1999. Plant determinants of mycorrhizal dependency in soybean. Agron. J. 91:135-141.

Klironomos, J.K., J. McCune, M. Hart, and J. Neville. 2000. The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecol. Lett. 3:137-141.

Koide, R.T., and M. Li. 1991. Mycorrhizal fungi and the nutrient ecology of three oldfield annual plant species. Oecologia 85:403-412.

Rousell, H., D. van Tuinen, P. Franken, S. Gianinazzi, and V. Gianinazzi-Pearson. 2001. Signalling between arbuscular mycorrhizal fungi and plants: identification of a gene expressed during early interactions by differential RNA display analysis. Plant Soil 232:13-19.

Smith, F.A., I. Jakobsen, and S.E. Smith. 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol. 147:357-366.

Smith, S., and D. Read. 1997. Mycorrhizal symbiosis. 2nd ed. Academic Press, London.

Streitwolf-Engel, R., T. Boller, A. Wiemken, and I.R. Sanders. 1997. Clonal growth traits of two *Prunella* species are determined by co-occuring arbuscular mycorrhizal fungi from a calcareous grassland. J. Ecol. 85:181-191.

van der Heijden, M.G.A., T. Boller, A. Wiemken, and I.R. Sanders. 1998a. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082-2091.

van der Heijden, M.G.A., J.K. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I.R. Sanders. 1998b. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature (London) 396:69-72.

van der Heijden, M.G.A., A. Wiemken, and I.R. Sanders. 2002. Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occuring plant. New Phytol. 157:569-578.

Appendix

Table 3A. Raw data for Table 3-1. Shoot dry weight at 4 wk.

G. mosseae				Control			
BSR201		Mandarin		BSR201		Mandarin	
Single	Mixed	Single	Mixed	Single	Mixed	Single	Mixed
g							
0.69	0.33	0.41	0.29	0.55	0.55	0.62	0.48
0.65	0.40	0.59	0.40	0.71	0.49	0.45	0.69
0.61	0.59	0.53	0.31	0.21	0.59	0.57	0.85
0.36		0.43		0.46		0.48	
0.10		0.51		0.22		0.39	
0.18		0.55		0.64		1.11	

Table 3B. Raw data for Table 3-1. Shoot dry weight at 8 wk.

G. mosseae				Control			
BSR201		Mandarin		BSR201		Mandarin	
Single	Mixed	Single	Mixed	Single	Mixed	Single	Mixed
g							
2.41	1.00	1.44	1.96	2.16	2.91	1.89	3.38
1.31	2.48	2.36	1.45	1.85	1.29	2.19	1.32
3.11	3.79	2.79	2.03	0.86	1.06	2.99	1.43
3.99		2.23		0.96		2.59	
1.77		2.54		2.09		2.26	
3.75		2.79		1.75		0.86	

Table 3C. Raw data for Table 3-1. Vegetative shoot dry weight at 8 wk.

G. mosseae				Control			
BSR201		Mandarin		BSR201		Mandarin	
Single	Mixed	Single	Mixed	Single	Mixed	Single	Mixed
g							
2.41	1.00	0.83	1.40	2.16	2.91	1.41	2.19
1.31	2.48	1.27	0.89	1.85	1.29	1.31	0.91
3.11	3.79	1.66	1.00	0.86	1.06	1.82	1.00
3.99		1.20		0.96		1.56	
1.77		1.80		2.09		1.40	
3.75		1.95		1.75		0.50	

Table 3D. Raw data for Fig. 3-1. Mycorrhizal colonization rates at 4 wk.

	BSR201						Mandarin					
	Single			Mixed			Single			Mixed		
rep.	1	2	3	1	2	3	1	2	3	1	2	3
	---------- % ----------											
	21	19	18	23	22	18	13	11	11	17	18	20
	22	23	27	20	18	18	11	11	9	12	16	15
	15	14	13	18	14	19	9	13	9	16	18	19
	14	16	15				10	9	9			
	17	13	15				9	10	11			
	19	15	15				10	8	10			

Table 3E. Raw data for Fig. 3-2. Mycorrhizal colonization rates at 8 wk.

	BSR201						Mandarin					
	Single			Mixed			Single			Mixed		
rep.	1	2	3	1	2	3	1	2	3	1	2	3
	---------- % ----------											
	53	55	52	48	51	50	45	42	42	50	49	48
	55	57	55	56	57	51	37	35	39	53	51	53
	50	48	55	49	55	51	44	41	37	51	51	50
	48	49	50				41	42	38			
	51	52	52				47	45	43			
	58	53	52				38	37	35			

CHAPTER 4. INTERACTIONS AMONG ARBUSCULAR MYCORRHIZAL FUNGI AND THEIR IMPACT ON SOYBEAN GROWTH

A paper prepared in the style for publication in Agronomy Journal

Satoshi Ishii and Thomas E. Loynachan

Abstract

Interactions among AM fungi and their impact on plant growth were examined. A conceptual model to explain functional complementarity/redundancy was constructed using the AM fungi with short hyphae, *Glomus caledonium* RIS42 (GC) and *Glomus mosseae* BEG83 (GM), and an AM fungus with long hyphae, *Scutellospora calospora* WUM12 (SC), and two soybean varieties, BSR201 and Mandarin, for AM fungal hosts. Infection activity of each fungal inoculum was set to be 10% by the Mean Infection Percentage Method prior to the main greenhouse experiment. Species within the roots were identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing.

AM fungal effects on growth of soybean cv. BSR201 were different among fungal inoculants, but differences were not observed in soybean cv. Mandarin. *Scutellospora calospora* WUM12 had extremely low colonization with somewhat negative effects in both BSR201 and Mandarin. Our initial conceptual model with three AM fungi did not function

as expected because of the low colonization activity of SC and contamination in the GC inoculum. In the GC inoculum, competition between AM fungi might exist because *G. caledonium* seemed to be excluded from the roots by the contaminant identified as *G. mosseae* by sequence analysis.

More research is necessary on the interaction between SC and the host plants to verify the concept of functional complementarity/redundancy between roots and hyphae and preferable host-fungus combinations proposed by Koide (2000). Also, the contaminant in the inoculum of GC should be eliminated for reliable research.

Introduction

Arbuscular mycorrhizal (AM) fungi help plants with their nutrient acquisition in return for receiving carbohydrate as their energy source (Smith and Read, 1997). Since AM fungi have broad host specificity, they could colonize together in the same host (van Tuinen et al., 1998). Many researchers reported the coexistence of several AM fungal species in the same roots (Clapp et al., 1995; Helgason et al., 1998; van der Heijden et al., 1998a, b; van Tuinen et al., 1998; Helgason et al., 1999; Jacquot et al., 2000; Daniell et al., 2001; Vandenkoornhuyse et al., 2002). For interactions among AM fungal species, however, both positive (mutualism) (van Tuinen et al., 1998; Jacquot et al., 2000) and negative (competition) (Wilson and Trinick, 1983; Abbott and Robson, 1984; Wilson, 1984; Hepper et al., 1988; Lopez-Aguillon and Mosse, 1987; Sainz et al., 1989; Daft, 1993) interactions have

been reported. More research is necessary to understand clearly the interactions among AM fungal species.

Impacts of coexistence of several AM fungal species on their host plants also have been documented (Streitwolf-Engel et al., 1997; van der Heijden et al., 1998a; 1998b, 2002). Although different impacts on the host plants were observed among three AM fungal species (all belonging to *Glomus*), mixed inoculation of these AM fungi did not increase plant biomass and nutrient acquisition compared with single inoculation of AM fungus (van der Heijden et al., 1998a; 2002). Different impacts on plant growth with inoculation are more apparent at the genus level than species or strain level (Hart and Reader, 2002; Klironomos and Hart, 2002). Colonization strategies such as colonization rate within plant roots, soil hyphal length, soil fungal biomass, source for infection, and infection speed also were different among AM fungal families (see Table 4-1; Hart and Reader, 2002; Klironomos and Hart, 2002). Therefore, study with other AM fungal genera and species may have different results on the significance of mycorrhizal diversity compared with the study by van der Heijden et al. (2002). The proportion of each fungus colonizing within the same roots (Jacquot et al., 2000), which was not examined by van der Heijden et al. (1998a; 1998b; 2002), might give us useful information to study the effects of AM fungal coexistence on their host plants, because mutualism, competition, or neutrality might occur among AM fungal species and affect the extent of colonization by each fungus.

Complementary or redundant function may be observed if different AM fungi with different colonization strategies are inoculated together (Koide, 2000; Smith et al., 2000; van der Heijden et al., 2002). Also, functional complementarity/redundancy might at least partially explain the different impacts of AM fungi among their plant hosts. Koide (2000)

proposed the concept that there might be preferable host-fungus combinations based on functional complementarity in nutrient acquisition between plant roots and AM fungal hyphae.

Based on the information previously reported, we constructed a conceptual model using three AM fungi, *Glomus caledonium* RIS42, *G. mosseae* BEG83, and *Scutellospore calospora* WUM12, which were shown to have different hyphal length and different nutrient acquisition efficiency (Jakobsen et al., 1992a, b; Pearson and Jakobsen, 1993a, b; Smith et al., 2000), and examined the impact of different AM fungal treatments on soybean growth and the interactions among different AM fungi using DNA-based molecular identification techniques to estimate the proportion of each fungus colonizing in the roots.

Materials and Methods

Soil preparation

The same soil was used as previously described. Soil preparation and characteristics of the soil sample were described in Chapter 2.

Mycorrhizal inoculum and infection assay

Three AM fungi, *Glomus caledonium* (Nicol. & Gerd.) Trappe & Gerd. (isolate RIS42 same as BEG15 and DN968; GC in this study), *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (isolate BEG83 same as DN990; GM in this study), and *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders (isolate WUM12 same as AU212; SC in this

study) were obtained from the International Culture Collection of Arbuscular and VA Mycorrhizal Fungi (INVAM), Morgantown, WV. Hyphal length and ability to acquire P were shown to be different between the two AM fungi, GC and SC (Jakobsen et al., 1992a, b; Pearson and Jakobsen, 1993a, b; Smith et al., 2000). Taxonomy-based differences in the colonization strategy of AM fungi are shown in Table 4-1, in which GC and GM belong to *Glomaceae*, whereas, SC belongs to *Gigasporaceae*.

The isolate GM was contributed to the INVAM by the same group who contributed GC and SC. The species *G. mosseae* is phylogenetically close to *G. caledonium*, and both belong to *Glomaceae*. These fungi have short hyphae and colonize roots rapidly and extensively (Table 4-1; Hart and Reader, 1997); therefore, we assumed that these isolates have ability to acquire nutrients and hypothesized that GC and GM were functionally redundant of each other when inoculated together (see Fig. 1-1A in Chapter 1). We also hypothesized that when GC and SC, GM and SC, or all the three fungi were inoculated together, their functions in nutrient acquisition complement each other (see Fig. 1-1B in Chapter 1). This was the primary reason we used the three AM fungi. *Glomus spp.*, especially *G. mosseae*, are commonly found in Iowa soil (Khalil et al., 1994; Troeh and Loynachan, 2003), and therefore, the interaction between *G. mosseae* and *G. caledonium* was of interest.

Initial inocula were propagated with soybean (*Glycine max* [L.] Merr.) cv. Mandarin for 10 wk in the greenhouse. Growth conditions were described in Chapter 2.

Table 4-1. Taxonomy-based differences in colonization strategy of AM fungi. This table was constructed based on the articles by Hart and Reader (2002) and Klironomos and Hart (2002).

	Gigasporaceae	Glomaceae
Colonization in roots	low	high
Soil hyphal length	long	short
Soil fungal biomass	high	low
Infection speed	slow	rapid
Sources for infection	spores	spores, hyphae, & mycorrhizal roots

Mean Infection Percentage (MIP)

Infection activity of each AM fungus was assayed by the Mean Infection Percentage Method (Moorman and Reeves, 1979). Briefly, each AM fungal inoculum was diluted 10-, 25-, and 50- fold with soil: sand mixture (60:40 by weight). Fungi were grown in the greenhouse with soybean cv. Mandarin for 4 wk. The growth conditions were described in Chapter 2. After 4-wk growth, plant roots were harvested, rinsed with sterile distilled water, and stained with 0.05% (w/v) trypan blue in lactoglycerol following the protocol by Brundrett et al. (1996) with minor modification, which is described briefly in Chapter 2. Stained soybean roots were cut into one-cm fragments, and mycorrhizal colonization rates were measured by the Gridline Intersect Method (Giovannetti and Mosse, 1980) under 40 × magnification using 0.25-0.30 g (fresh weight) of the root fragments. The linear regression equations between inoculum dilution and mycorrhizal colonization rate were constructed. Based on the equations, the infection activities, colonization 4 wk after inoculation in this study, were set to be 10% among the three AM fungi for the main greenhouse experiment.

Plant culture and the greenhouse experiment

Two soybean cultivars, BSR201 and Mandarin, were obtained from the Iowa Crop Improvement Association, Ames, IA. Their characteristics were described in Chapter 3.

The experimental design of this study is shown in Table 4-2. Initially, all combinations among three AM fungi, two soybean varieties, and two harvest times were planned, but due to the small amount of inocula, we changed our design as shown in Table 4-2. The treatments of most interest were GCGM, GCSC, and GMSC.

Table 4-2. Experimental design, with number of pots (number of plants) shown. Treatment GC was inoculated with *G. caledonium* RIS42; GM, with *G. mosseae* BEG83; SC, with *S. calospora* WUM12; GCGM, with *G. caledonium* RIS42 and *G. mosseae* BEG83; GCSC, with *G. caledonium* RIS42 and *S. calospora* WUM12; GMSC, with *G. mosseae* BEG83 and *S. calospora* WUM12; GCGMSC, with all the three AM fungi; and Control, without any inocula. The infection activities of all treatments except Control were set to be 10% based on MIP results.

Treatment	Mandarin		BSR201		Mandarin + BSR201	
	4 wk	8 wk	4 wk	8 wk	4 wk	8 wk
GC		2 (4)		2 (4)		2 (4)
GM		2 (4)		2 (4)		2 (4)
SC		2 (4)		2 (4)		2 (4)
GCGM	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)
GMSC	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)
SCGC	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)
GCGMSC		3 (6)		3 (6)		3 (6)
Control	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)	3 (6)

Soybean seeds were surface disinfected by soaking in 70% ethanol for 5 min, and rinsed with sterilized water five times. Seeds were pregerminated in sterile distilled water for 2 d. Two seeds of each soybean cultivar were planted in a 15-cm pot containing 2 kg soil: sand mixture. One seed each of the two cultivars was also planted together in the same pot.

They were grown for 4 and 8 wk in the greenhouse. Pots were arranged in a completely randomized design. The growth conditions were described in Chapter 2.

Plant harvesting

Four and 8 wk after planting, soybean shoots and roots were harvested. Shoots were oven-dried in paper bags at 105°C overnight, and their dry weights were recorded. Both BSR201 and Mandarin were at early vegetative stage (V3) after 4-wk growth, but their growth stages were different after 8-wk growth. BSR201 was at early reproductive stage (R2), in which flowering was started at all nodes. Mandarin was at early-to-late reproductive stage (R5), in which seeds were beginning to form.

Plant roots were gently washed in distilled water to remove soil particles and stained with 0.05% (w/v) trypan blue in lactoglycerol following the protocol by Brundrett et al. (1996) with minor modification (see Chapter 2).

Mycorrhizal colonization rate

Soybean roots stained with trypan blue were cut into one-cm fragments. Mycorrhizal colonization rates were measured by the Gridline Intersect Method (Giovannetti and Mosse, 1980) using 0.25-0.30 g (fresh weight) of the root fragments.

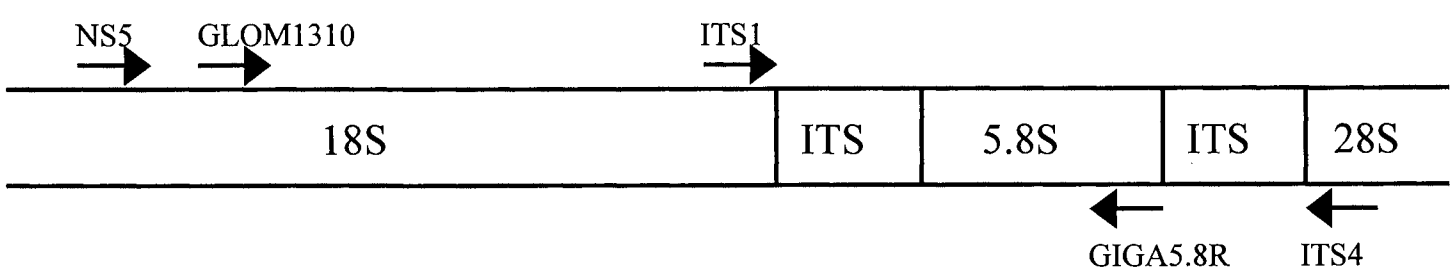
DNA extraction and PCR

DNA was extracted from trypan blue-stained mycorrhizal roots by bead beating with Chelex-100 purification as described in Chapter 2. Ribosomal RNA region of AM fungal DNA was amplified by nested-PCR using eukaryotic universal primers NS5 and ITS4 (White et al., 1990) in the first step. In the second step, *G. mosseae* and/or *G. caledonium* DNA was

Table 4-3. Primers used in this study.

Name	Direction	Sequence (5'-3')	Target group	Reference
NS5	Forward	AACTTAAAGGAATTGACGGAAG	Eukaryotes	White et al., 1990
ITS4	Reverse	TCCTCCGCTTATTGATATGC	Eukaryotes	White et al., 1990
ITS1	Forward	TCCGTAGGTGAACCTGCGG	Eukaryotes	White et al., 1990
GLOM1310	Forward	AGCTAGGCTTAACATTGTTA	*Glomus mosseae /intraradices* group	Redecker, 2000
GIGA5.8R	Reverse	ACTGACCCTCAAGCATGTG	*Gigasporaceae*	Redecker, 2000
T7	Forward	AATACGACTCACTATAG	pGEM-T or other cloning vectors	Promega, Madison, WI
SP6	Reverse	ATTTAGGTAGCACTATAG	pGEM-T or other cloning vectors	Promega, Madison, WI

Fig. 4-1. Annealing sites of primers on ribosomal RNA genes. Drawing is not to scale.



amplified with GLOM1310, a primer specific to *Glomus mosseae/intraradices* group (Redecker, 2000), in combination with ITS4 primer. *S. calospora* DNA was amplified with GIGA5.8R, a primer specific to *Gigasporaceae* (Redecker, 2000) in combination with NS5 primer, in the second step. These primers were synthesized by Integrated DNA Technologies (Coralville, IA). Sequences and annealing sites of the primers used in this study are shown in Table 4-3 and Figure 4-1. PCR conditions were described in Chapter 2.

DNA was also extracted from AM fungal spores. AM fungal spores were collected by the method of Adelman and Morton (1986) and Troeh and Loynachan (2003). DNA was extracted from AM fungal spores following the protocol by van Tuinen et al. (1998), and amplified with the primers Glom1310 and ITS4 for the DNA from GC and GM (both belong to *Glomus mosseae/intraradices* group) spores and with the primers Giga5.8R and NS5 for SC (belongs to *Gigasporaceae*) spores. Double-step PCR or nested-PCR was not applied in this step because the extract from an AM fungal spore contains enough DNA for one-step PCR. The same PCR conditions were applied as descried in Chapter 2.

Restriction analysis

Amplified DNA was cut into several pieces by the restriction endonuclease, *Hinf* I or *Dpn* II. Eight μL of PCR product was digested in 15 μL of reaction mix containing 10 U of restriction endonuclease. Fragment patterns were analyzed on 4% (w/v) agarose gel, stained with ethiduim bromide, and photographed.

Cloning and sequencing

For sequence analysis, third step PCR was performed using ITS4 and ITS1. The amplified product of this step contains highly variable internal transcribed spacers (Fig. 4-1) with short length (approximately 600 bp) that is a convenient length for cloning and sequencing. The amplified product of the second step PCR was diluted 1/1000, and 1 μL was used as DNA template in third reaction containing the same reaction mixture with the total volume of 25 μL. Annealing temperature was 53°C for 25 cycles. Aliquots of 3 μL were run on a 2% (w/v) agarose gel and stained with ethidium bromide to confirm the DNA amplification of estimated size (approximately 600 bp).

DNA amplification with ITS4 and ITS1 was also performed from spore samples. DNA was amplified directly from the DNA extracted from spores (neither double-step PCR nor nested-PCR was applied) using the same conditions described above except with 35 cycles.

Amplified DNA fragments were recovered and purified from the 2% (w/v) low-melting agarose gel using Zymoclean Gel DNA Recovery Kit (ZYMO Research, Orange, CA), cloned into pGEM-T Vector (Promega, Madison, WI), and transformed into *Escherichia coli* DH5α following the manufacturer's instructions. Plasmid inserts were checked using PCR with T7 and SP6 primers, which correspond to pGEM plasmid sequences (Table 4-2). In this step, white colonies on LB plates were picked with toothpicks directly into the PCR reactions containing 0.2 m*M* of each dNTP, 0.5 μ*M* of each primer, 1.5 m*M* of $MgCl_2$, 0.02 U μL of Platinum*Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and the manufacturer's reaction buffer. Small scale reactions with the total volume of 7.5 μL were used to minimize reaction costs.

From each transformed sample, three bacterial colonies containing the vector with insert were incubated overnight at 37°C, and plasmid DNA was extracted using UltraClean™ 6 Minute Mini Plasmid Prep Kit (MoBio, Solana Beach, CA) and sequenced with T7 primer using ABI PRISM® 377 Sequencer (Applied Biosystems, Foster City, CA).

Sequence results were modified manually with the aid of computer software, Chromas 1.45 (available from www.technelysium.com.au/chromas.html) and GeneRunner version 3.05 (Hastings Software, Inc., Hastings-on-Hudson, NY). Modified sequences were identified using the BLAST program (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/). They were also aligned and shaded using GeneDoc version 2.6.002 (available from www.psc.edu/biomed/genedoc) to compare the sequences obtained in this study. Based on alligned sequences, a dendrogram was constructed using Neighbor-joining program in Phylip version 3.6 (available from http://evolution.genetics.washington.edu/phylip.html) to cluster our sequences and sequences from the database.

Statistical analyses

All numerical data were statistically analyzed by one-way ANOVA using SAS program. Colonization rates among plants and among pots were not significantly different; therefore, colonization rates were analyzed based on treatments. Scheffe's test was used to group treatments based on colonization rates.

Results

Soybean growth

Soybean seedlings grew healthy, with no symptoms of disease. Their growth was estimated by shoot dry weights shown in Fig. 4-2, 4-3, 4-4, and 4-5. No significant differences were found in shoot dry weights of both BSR201 and Mandarin 4 wk after planting (Fig. 4-2 and 4-3). After 8-wk growth, shoot dry weights of BSR201 were different among the treatments (Fig. 4-4). Fig. 4-4 indicated the tendency that GC and GM promoted soybean growth but SC did not; rather SC had a negative effect. Also, SC seemed to attenuate the positive effect of GC and GM, when inoculated together (GCSC, GMSC, and GCGMSC treatments, Fig. 4-4). These tendencies, however, were not strong; pair-wise comparisons based on Scheffe's test, which controls the type I experimental error rate, showed that the differences in shoot dry weights were significant only between SC and GCGM treatments. Statistical analysis also showed that shoot dry weights of Mandarin 8 wk after planting were not different among treatments (Fig. 4-5).

Mycorrhizal colonization rates

Mycorrhizal colonization rates 8 wk after planting were significantly different among treatments both in BSR201 and Mandarin (Fig. 4-6 and 4-7). Mycorrhizal colonization rates of the SC treatment were extremely low in all varieties, pots, and plants. In BSR201, Scheffe's test grouped GC, GCGM, GCSC, GMSC based on their mycorrhizal colonization rates; in other words, there were no significant differences in mycorrhizal colonization rates among GC, GCGM, GCSC, and GMSC. In Mandarin, Scheffe's test grouped GC, GCGM,

Fig. 4-2. Shoot dry weight of soybean cv. BSR201 4 wk after planting (mean ± SEM, n=6)

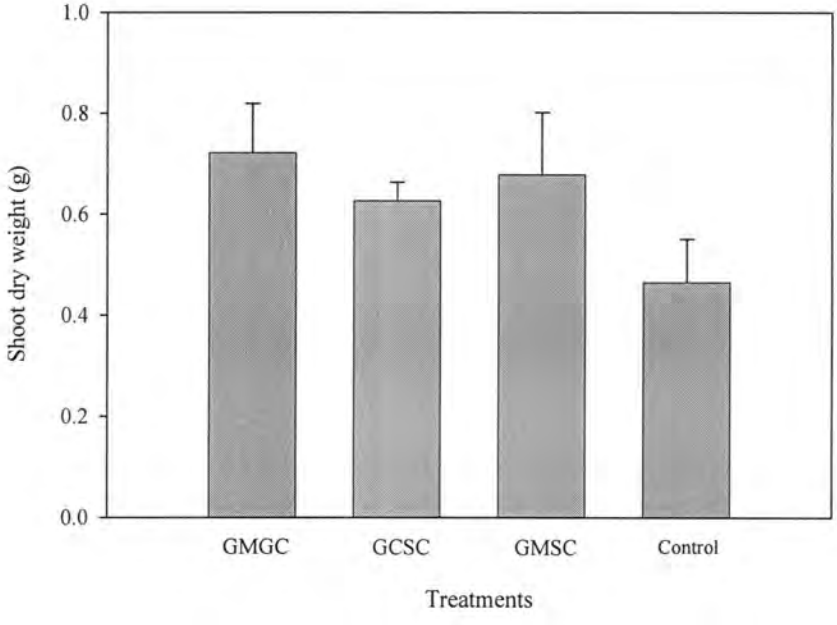


Fig. 4-3. Shoot dry weight of soybean cv. Mandarin 4 wk after planting (mean ± SEM, n=6)

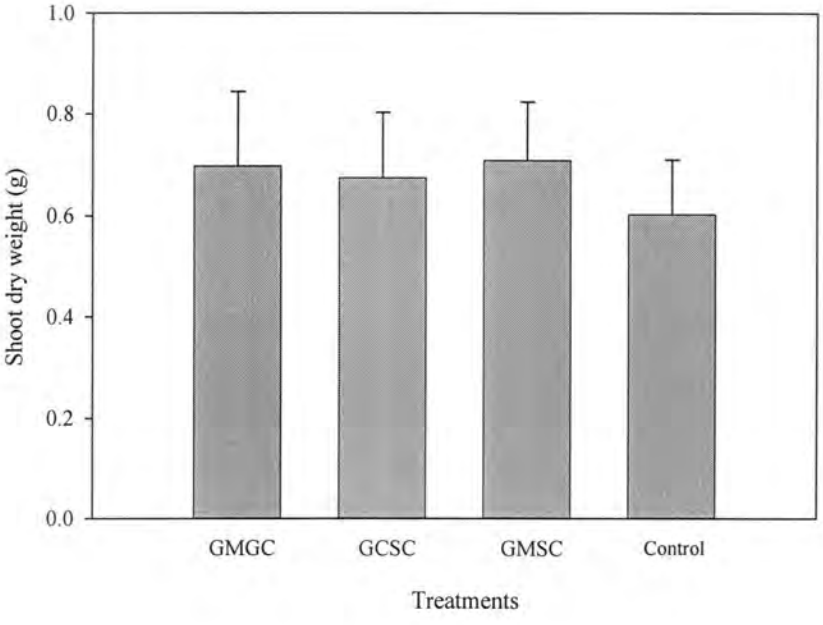


Fig. 4-4. Shoot dry weight of soybean cv. BSR201 8 wk after planting (mean ± SEM, n=6)

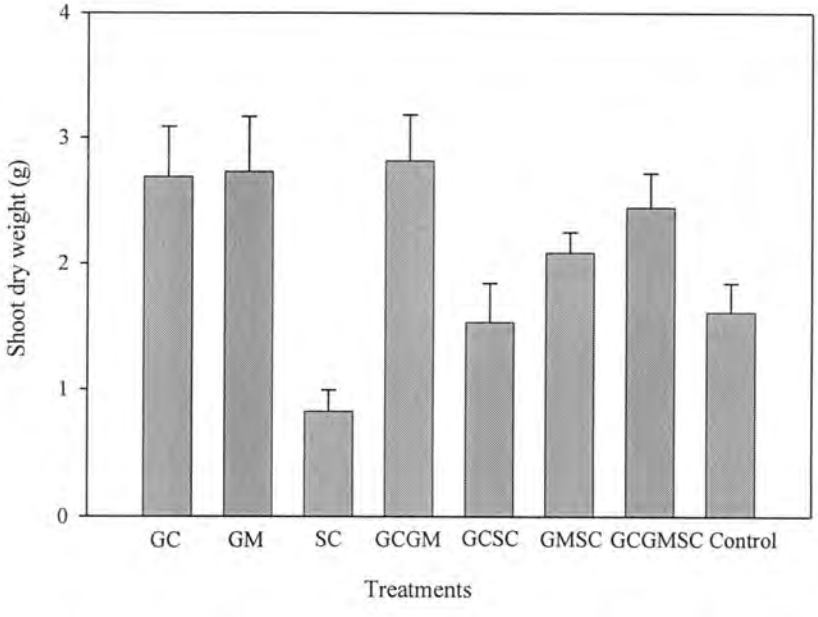


Fig. 4-5. Shoot dry weight of soybean cv. Mandarin 8 wk after planting (mean ± SEM, n=6)

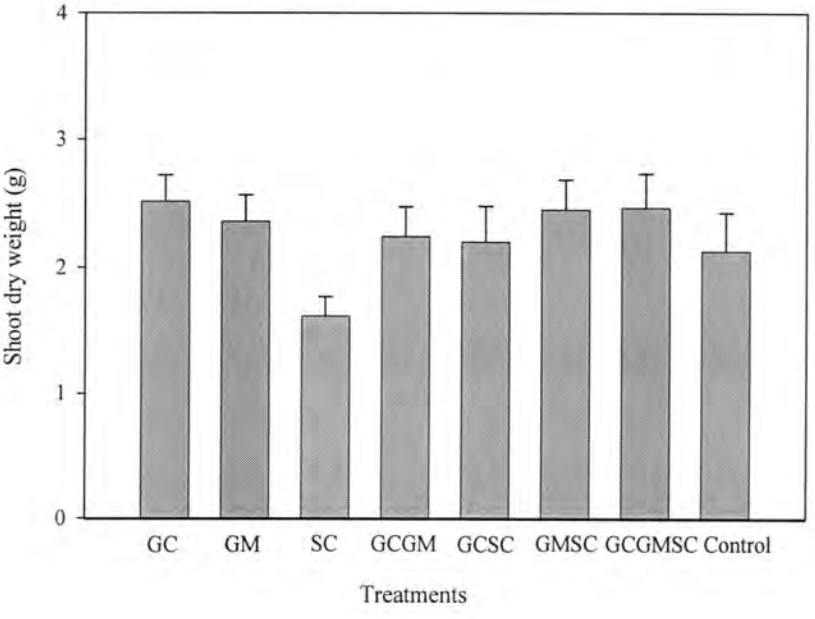


Fig. 4-6. Mycorrhizal colonization rates in soybean cv. BSR201 8 wk after planting (mean ± SEM, n = 12 for GC and SC, n = 18 for GM, GCGM, and control, and n=6 for GCSC and GMSC).

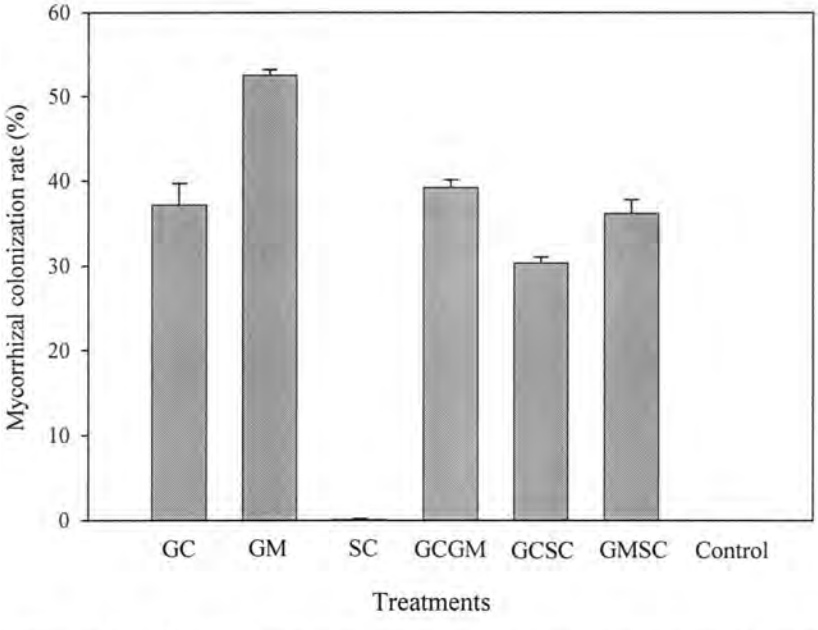
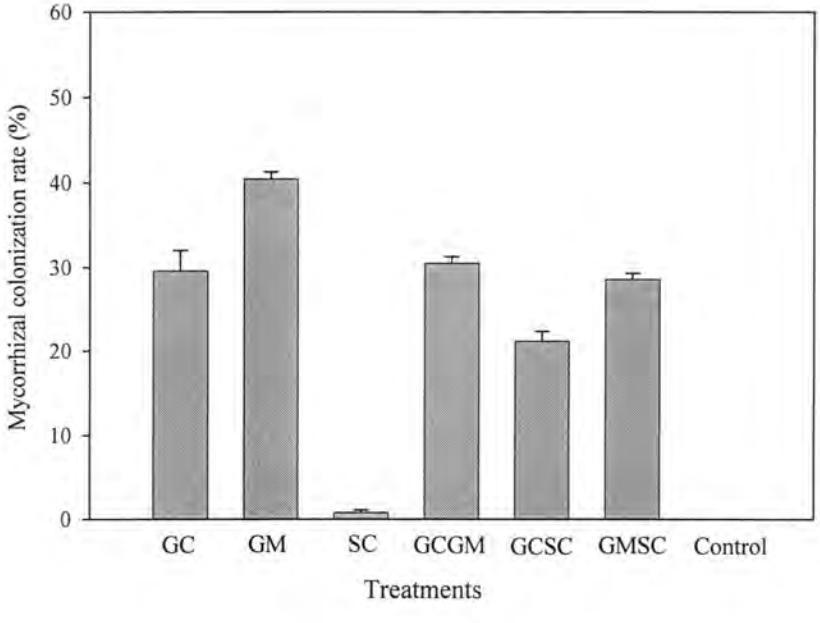


Fig. 4-7. Mycorrhizal colonization rates in soybean cv. Mandarin 8 wk after planting (mean ± SEM, n = 12 for GC and SC, n = 18 for GM, GCGM, and control, and n=6 for GCSC and GMSC).



and GCSC based on their mycorrhizal colonization rates. Similar tendencies in mycorrhizal colonization rates were obtained both from BSR201 and Mandarin, but mycorrhizal colonization rates were generally higher in BSR201.

DNA extraction and PCR-RFLP

DNA was successfully extracted from AM fungal spores as well as from mycorrhizal roots. PCR products obtained from spores and mycorrhizal roots are shown in Fig. 4-8 and 4-10, respectively. Based on Fig. 4-8, both GC and GM give almost the same length of band (approximately 1000 bp); therefore, in order to distinguish between GC and GM, restriction analysis was necessary.

Amplified products obtained from spores were digested with one of the two restriction endonucleases, *Hinf* I or *Dpn* II. The RFLP patterns of GC and GM are shown in Fig. 4-9. The RFLP patterns of the amplified products from the mixture of DNA extract of GC and GM spores are also shown in Fig. 4-9. Fig. 4-9 shows that both *Hinf* I and *Dpn* II are useful to distinguish between GC and GM. When both GC and GM existed in the samples, the RFLP pattern of such mixture showed identical bands to both GC and GM, no matter which restriction endonuclease was used (Fig. 4-9). Fig. 4-9 also suggested that the DNA of GC and GM were almost equally amplified when they were mixed together.

Almost the same length (approximately 1000 bp) of PCR products were obtained from spores (Fig. 8) and mycorrhizal roots (Fig. 10). PCR products shown in Fig. 10 came from GCGM treatment. In order to identify the AM fungal species colonizing roots, these PCR products were digested with *Hinf* I and run on 4% (w/v) agarose gel (Fig. 11). Although the RFLP patterns (lane 2-8) in Fig. 11 were a little faint, at least two or three

Fig. 4-8. PCR products obtained from AM fungal spores on 1.2% (w/v) agarose gel. Lane 1, 100 bp DNA ladder (from bottom to top, 600, 700, 800, 900, 1000, 1100, 1200, and 1300 bp); lane 2-4, GM; lane 5-7, GC. GM spores used here were small (roughly 100-200 μm) and brown, and GC spores used here were big (roughly 200-300 μm) and brown (see Table 4-4).

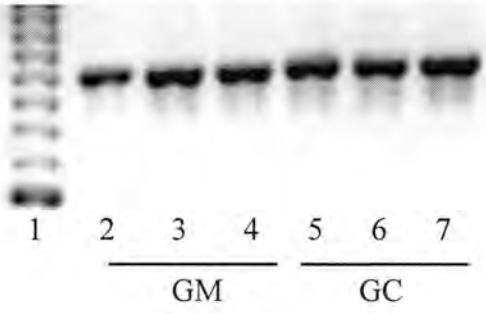


Fig. 4-9. RFLP pattern obtained from AM fungal spores on 4% (w/v) agarose gel. Lane 1 and 8, 100 bp DNA ladder (from bottom to top, 100, 200, 300, 400, 500, and 600 bp); lane 2-4, digested with *Dpn* II; lane 5-7, digested with *Hinf* I, lane 2 and 5, GC; lane 3 and 6, GM, lane 4 and 7, amplified product from the mixture of DNA extract of GC and GM.

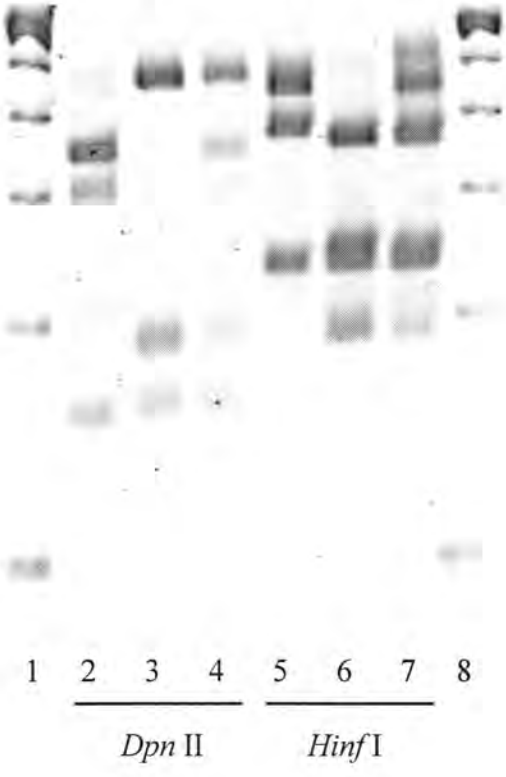
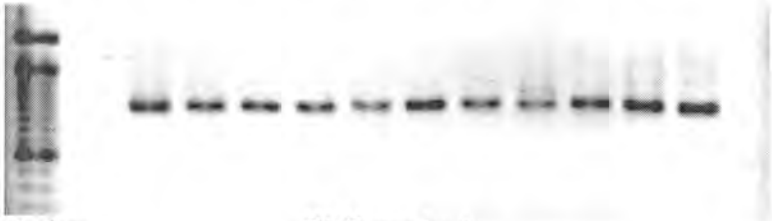
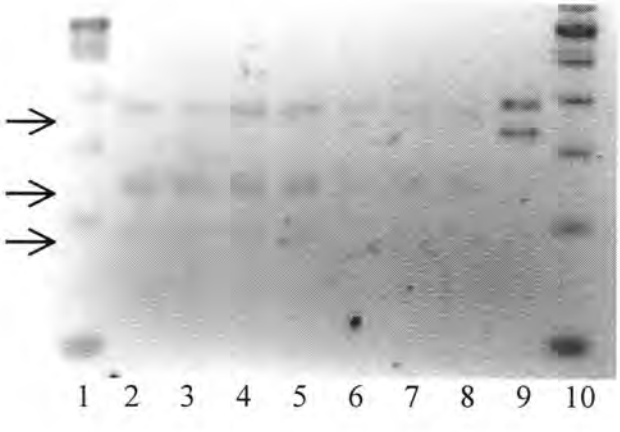


Fig. 4-10. Successful amplification of AM fungal DNA extracted from stained mycorrhizal roots on 1.2% (w/v) agarose gel. The lane far left shows DNA marker (100 bp DNA ladder, three bold bands indicate 600, 1500, and 2000 bp, from bottom to top). PCR products were from GCGM samples.



Marker PCR products

Fig. 4-11. Example of RFLP results obtained from mycorrhizal root samples on 4% (w/v) agarose gel. Samples were digested with *Hinf* I. Lane 1 and 10, 100 bp DNA ladder (from bottom to top, 100, 200, 300, 400, 500, and 600 bp); lane 2-8, GCGM; lane 9, SC. Three arrows indicate the identical bands to GM (see Fig. 4-7).



identical bands to GM (from bottom to top, approximately 180, 250, and 360 bp) were observed. No identical bands to GC except 250 bp were observed in Fig. 11. All RFLP analysis we tried indicated that only GM colonized within the roots of GCGM treatment.

Further, even from the mycorrhizal roots in GC treatment, we mostly detected GM, suggesting there was a contaminant in the GC inoculum. The lane 9 in Fig. 11 was the RFLP pattern from the mycorrhizal roots in SC treatment, and this pattern was identical to the RFLP pattern obtained from SC spores (data not shown).

Cloning and sequencing

Since we observed a possible contamination in GC inoculum, we tried to identify the contaminant by sequencing. First, DNA was amplified with ITS1 and ITS4 primers (Table 4-3 and Fig. 4-1) both from spores in GC and GM inocula and from mycorrhizal roots in GC and GM treatments (Fig. 4-12). All amplified products had approximately 600 bp (Fig. 4-12). Their RFLP patterns are shown in Fig. 4-13. Except GC12 and GC13 (lane 3 and 4 in Fig. 13), all samples had the same RFLP patterns. Spore morphology of GC1 was similar to GM8-10 (small [roughly 100-200 μm], brown, and globular spores), while GC12 and GC13 were big (roughly 200-300 μm), brown, and somewhat oval spores.

Fig. 4-12. PCR products amplified with ITS1 and ITS4 on 2% (w/v) agarose gel. Lane 1, 100 bp DNA ladder (from bottom to top, 400, 500, 600, 700, 800, and 900 bp); lane 2, GC1; lane 3, GC12; lane 4, GC13; lane 5, GC8M1; lane 6, GC8M2, lane 7, GC8M3; lane 8, GM1; Lane 9, GM2; lane 10, GM3; lane 11, GM8M1; lane 12, GM8M2; lane 13, GM8M3.

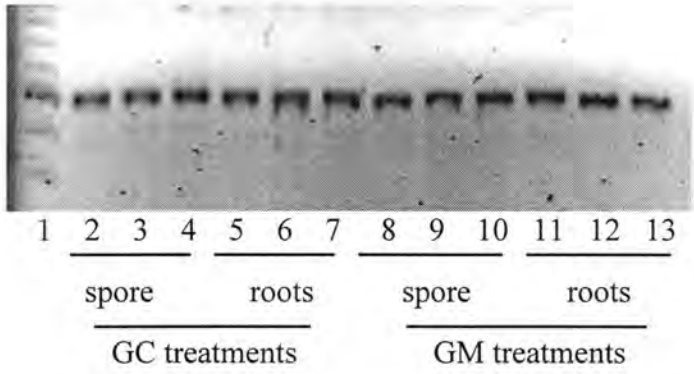


Fig. 4-13. PCR products shown in Fig. 12 were digested with *Hinf* I and run on 4% (w/v) agarose gel. Lane 1 and 14, 100 bp DNA ladder (from bottom to top, 100, 200, 300, 400, 500, and 600 bp); lane 2, GC1; lane 3, GC12; lane 4, GC13; lane 5, GC8M1; lane 6, GC8M2; lane 7, GC8M3; lane 8, GM1; lane 9, GM2; lane 10, GM3; lane 11, GM8M1; lane 12, GM8M2; lane 13, GM8M3. Spore morphology of GC1, and GM8-10 were similar (small brown globular spores; see Table 4-4).

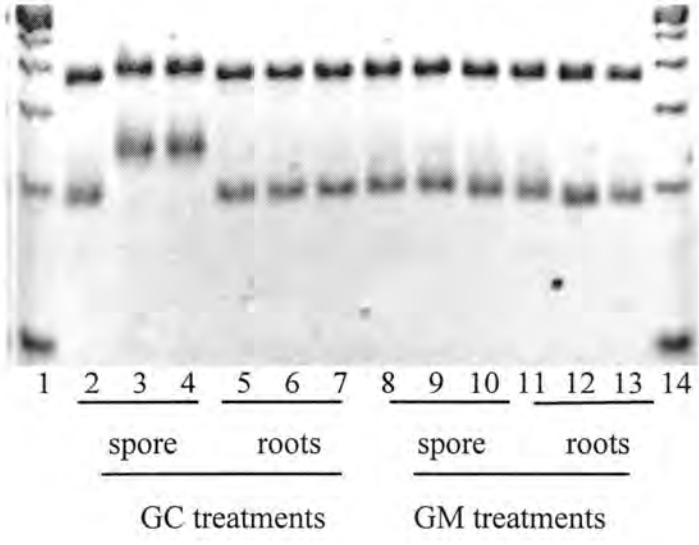
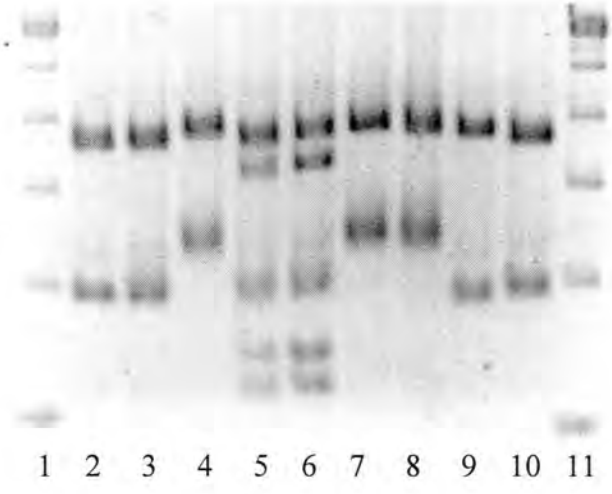


Fig. 4-14. PCR products with ITS1 and ITS4 primers from spore samples were digested with *Hinf* I and run on 4% (w/v) agarose gel. Lane 1 and 11, 100 bp DNA ladder (from bottom to top, 100, 200, 300, 400, 500, and 600 bp); lane 2, GC2; lane 3, GC3; lane 4, GC4; lane 5, GC5; lane 6, GC6; lane 7, GC7; lane 8, GC8; lane 9, GC9; lane 10, GC11. Morphology of these spores are shown in Table 4-4.



More spores were examined to see the relationship between spore morphology and RFLP patterns (Fig. 4-14 and Table 4-4). Spores were grouped based on their morphology and RFLP patterns, and all groups were cloned and sequenced (Table 4-4). ITS region of ribosomal DNA extracted and amplified from mycorrhizal roots in GC (GC8M1) and GM (GM8M1) were also cloned and sequenced.

Table 4-4. Morphology and RFLP pattern of AM fungal spores. A small spore was roughly 100-200 μm, and a big spore was roughly 200-300 μm. Spores were grouped based on their morphology and RFLP patterns.

Sample	Spore morphology			RFLP pattern		Sequence	Source
	Color	Size	Group	Picture	Group		
GC1	brown	small	a	lane 2 on Fig. 4-13	A	GC1A, B, C	Our inoculum
GC2	brown	small	a	lane 2 on Fig. 4-14	A		Our inoculum
GC3	brown	small	a	lane 3 on Fig. 4-14	A		Our inoculum
GC4	brown	big	b	lane 4 on Fig. 4-14	B		INVAM culture
GC5	brown	small	a	lane 5 on Fig. 4-14	C	GC5A, B, C	INVAM culture
GC6	brown	small	a	lane 6 on Fig. 4-14	C		INVAM culture
GC7	brown	big	b	lane 7 on Fig. 4-14	B	GC7A, B, C	INVAM culture
GC8	brown	big	b	lane 8 on Fig. 4-14	B		INVAM culture
GC9	white	small	c	lane 9 on Fig. 4-14	A	GC9A, B, C	INVAM culture
GC11	white	small	c	lane 10 on Fig. 4-14	A	GC11A, B, C	INVAM culture
GC12	brown	big	b	lane 3 on Fig. 4-13	B		INVAM culture
GC13	brown	big	b	lane 4 on Fig. 4-13	B		INVAM culture
GM1	brown	small	a	lane 8 on Fig. 4-13	A	GM1A, B, C	Our inoculum
GM2	brown	small	a	lane 9 on Fig. 4-13	A		Our inoculum
GM3	brown	small	a	lane 10 on Fig. 4-13	A		Our inoculum

Amplified products of ITS region of ribosomal DNA was successfully inserted into the pGEM-T vector and transformed into *E. coli*. Fig. 4-15 shows the results of plasmid insert check using PCR with T7 and SP6 primers. PCR products shown in Fig. 4-15 had approximately 760 bp as expected (approximately 600 bp of PCR insert plus 160 bp of the vector sequences) except GC5A and GC5C.

Fig. 4-15. Results of plasmid insert check by PCR with T7 and SP6 primers. Amplified products were run on 2% (w/v) agarose gel. Lane 1, 14, 15, and 28, 100 bp DNA ladder (from bottom to top, 600, 700, 800, and 900 bp); lane 2, GC1A; lane 3, GC1B; lane 4, GC1C; lane 5, GC8M1A; lane 6, GC8M1B; lane 7, GC8M1C; lane 8, GM1A; lane 9, GM1B; lane 10, GM1C; lane 11, GM8M1A; lane 12, GM8M1B; lane 13, GM8M1C; lane 16, GC5A; lane 17, GC5B; lane 18, GC5C; lane 19, GC7A; lane 20, GC7B; lane 21, GC7C; lane 22, GC9A; lane 23, GC9B; lane 24, GC9C; lane 25, GC11A; lane 26, GC11B; lane 27, GC11C.

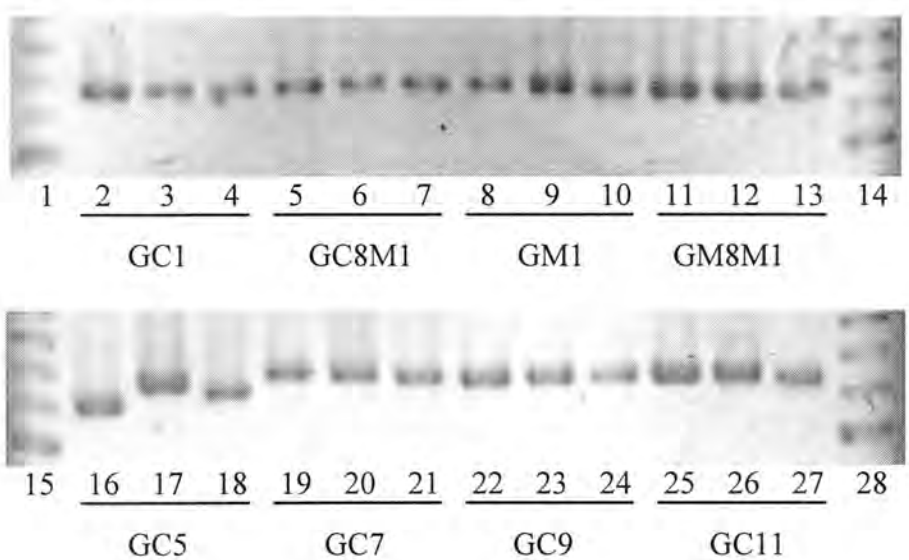


Table 4-5. Species identification based on sequence result and BLAST searching.

Sequences from this study	Sequence from database	Accession	Identity with the database sequence (%)	Base length compared with the database (bp)
GC1A	*G. mosseae* BEG12	X84233	97.4	583
GC1B	*G. mosseae* BEG12	X84233	98.1	581
GC1C	*G. mosseae* Nr 423	AY035650	97.1	581
GC8M1A	*G. mosseae* BEG12	X84233	96.1	583
GC8M1B	*G. mosseae* BEG12	X84233	96.1	583
GC8M1C	*G. mosseae* BEG12	X84233	96.1	583
GM1A	*G. mosseae* Nr 423	AY035650	97.6	583
GM1B	*G. mosseae* BEG12	X84233	96.6	583
GM1C	*G. mosseae* BEG61	X96836	98.2	562
GM8M1A	*G. mosseae* FL156	U49265	98.1	527
GM8M1B	Luciferase gene	AF416990	97.3	691
GM8M1C	*G. mosseae* BEG12	X84233	98.1	581
GC5A	*G. mosseae* Nr 423	AY035650	97.6	531
GC5B	*G. mosseae* Nr 423	AY035650	96.7	584
GC5C	*Gastrostyla steinii*	AF508758	98.8	564
GC7A	*G caledonium* clone JJ36	AY035642	98.8	600
GC7B	*G. caledonium* Nr 658	AY035651	98.8	602
GC7C	*G. caledonium* clone JJ40	AY035646	99.0	599
GC9A	*G. mosseae* BEG12	X84233	95.9	583
GC9B	*G. mosseae* Nr 423	AY035650	97.0	571
GC9C	*G. mosseae* BEG12	X84233	95.9	583
GC11A	*G. mosseae* BEG12	X84233	97.4	582
GC11B	*G. mosseae* BEG61	X96836	97.9	563
GC11C	*G. mosseae* FL156	U49265	98.9	536

Species identification based on sequence results and the BLAST searching is shown in Table 4-5. All clones except GM8M1B, GC5C, GC7A, B, and C were identical with *G. mosseae*. GC7A, B, and C were identical with *G. caledonium*. A dendrogram constructed based on sequences from this study (except GM8M1) and sequences from the database

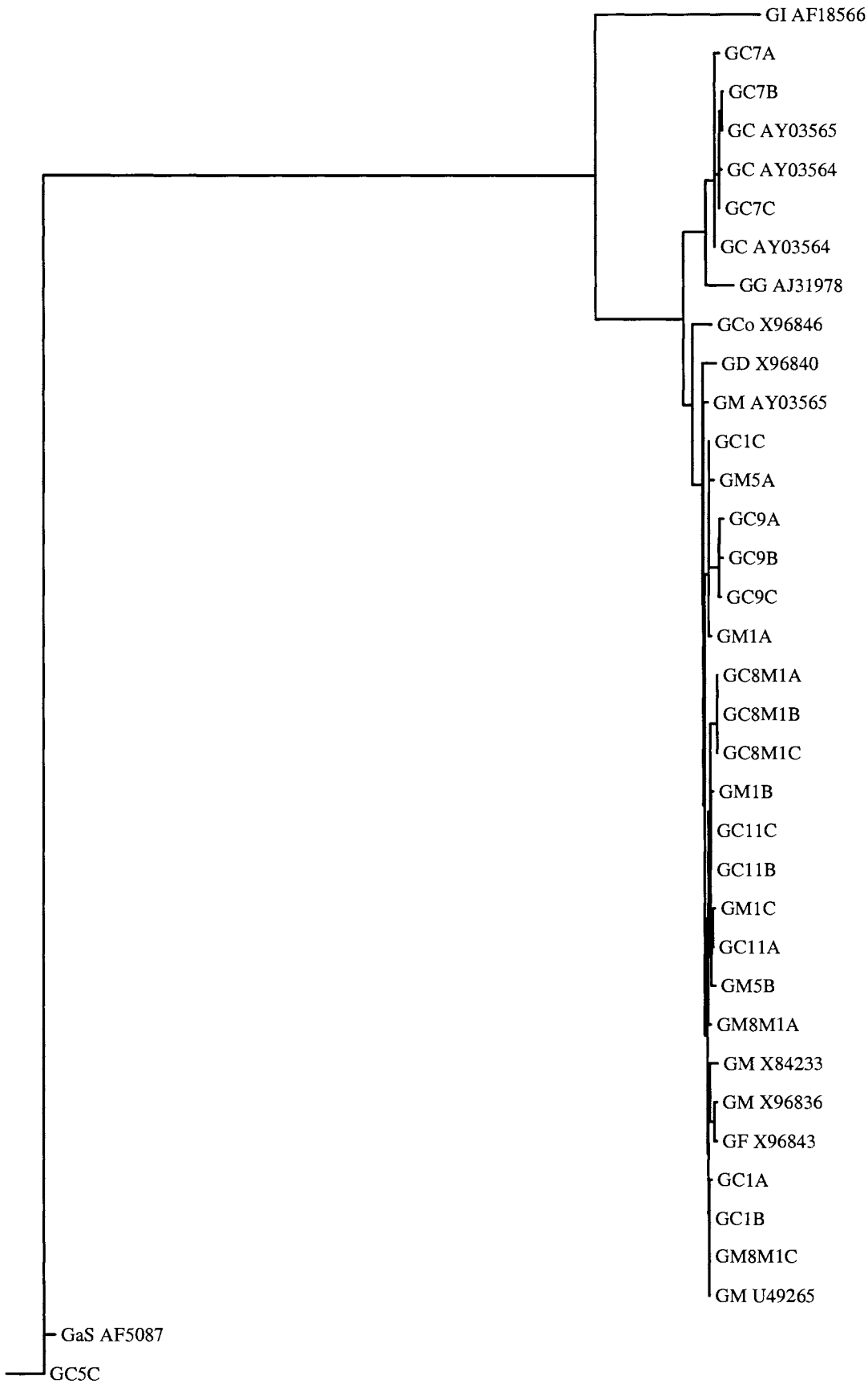
(Table 4-6) is shown in Fig. 4-16. In Fig. 4-16, *G. mosseae* and *G. caledonium* was clustered separately, supporting the species identification described above.

Table 4-6. Sequences from the database used to construct a dendrogram shown in Fig. 4-16.

Sequence from the database	Species name
GC_AY035651	*G. caledonium* Nr 658
GC_AY035642	*G. caledonium* JJ36
GC_AY035646	*G. caledonium* JJ40
GCo_X96846	*G. coronatum* BEG28
GD_X96840	*G. dimorphicum* BEG59
GG_AJ319780	*G. geosporum*
GI_AF185663	*G. intraradices* FL208
GM_X844233	*G. mosseae* BEG12
GM_U49265	*G. mosseae* FL156
GM_AY035650	*G. mosseae* Nr 243
GM_X96836	*G. mosseae* BEG61
GaS_AF508758	*Ga. steinii* (outgroup)

The sequences identified as *G. mosseae* and *G. caledonium* were aligned in Fig. 4-17 and 4-18, respectively. Bases not common among the clones are shaded with gray in Fig. 4-17 and 4-18. Also, *Hinf* I recognition sites (5'-GANTC-3') are shaded with black. In Fig. 4-17, bases are numbered on the top up to 585, in which 1-30 codes for partial 18 S rRNA, 31-162 for ITS1, 163-301 for 5.8 S rRNA, 302-527 for ITS2, and 528-585 for partial 28 S rDNA. The sequences of primers ITS1 and ITS4 are shown from 1-19 and from 566-585, respectively. In Fig. 4-18, bases are also numbered on the top up to 606, in which 1-30 codes for partial 18 S rRNA, 31-167 for ITS1, 168-305 for 5.8 S rRNA, 306-548 for ITS2, and

Fig. 4-16. A dendrogram constructed based on sequences from this study (except GM8M1) and sequences from the database (Table 4-6).


GI AF18566
GC7A
GC7B
GC AY03565
GC AY03564
GC7C
GC AY03564
GG AJ31978
GCo X96846
GD X96840
GM AY03565
GC1C
GM5A
GC9A
GC9B
GC9C
GM1A
GC8M1A
GC8M1B
GC8M1C
GM1B
GC11C
GC11B
GM1C
GC11A
GM5B
GM8M1A
GM X84233
GM X96836
GF X96843
GC1A
GC1B
GM8M1C
GM U49265
GaS AF5087
GC5C

549-606 for partial 28 S rDNA. The sequences of primers ITS1 and ITS4 are shown from 1-19 and from 587-606, respectively. In both Fig. 4-17 and 4-18, more sequence variations were observed in ITS1 and ITS2 region than 18 S, 5.8 S, and 28 S rRNA genes.

Based on *Hinf* I recognition sites, restriction fragment length of PCR products amplified with ITS1 and ITS4 were calculated (Table 4-7). Restriction fragment length of *G. caledonium* and *G. mosseae* were compatible with the RFLP patterns shown in Fig. 4-13 and 4-14.

GC5C was identical with partial 17 S rRNA, ITS1, 5.8 S rRNA, ITS2, and partial 26 S rRNA genes of *Gastrostyla steinii* (98.8%), *Oxytricha trifallax* (94.4%), or *O. fallax* (93.3%). All *Ga. steinni*, *O. trifallax*, and *O. fallax* belong to the family, *Oxytrichidae* (ciliates). Sequence alignment of GC5A, *Ga. steinii*, *O. trifallax*, *O. fallax* is shown in Fig. 4-19. Bases different from GC5C sequences are shaded with gray. The sequences shaded with black indicate *Hinf* I recognition sites. GC5A had the common Hinf I recognition sites with *Ga. steinni*, but not with *O. trifallax* or *O. fallax*; therefore, GC5A was identified as *Ga. steinni*. Restriction fragment length of *Ga. steinii* DNA amplified with ITS1 and ITS4 were shown in Table 4-6. Both the RFLP pattern of GC5 (lane 5 in Fig. 4-14) and restriction fragment length (Table 4-7) suggested that GC5 contained both *G. mosseae* and *Ga. steinni*.

Table 4-7. Restriction fragment length based on the sequences of *G. caldonium*, *G. mosseae*, and *Ga. steinii*. Means of each species are shown. Variation within species was small (less than ± 2 bp).

Species	Restriction fragment length (bp)	Uncut fragment length (bp)
G. caledonium	368, 232	600
G. mosseae	352, 183, 45	580
Ga. steinii	315, 137, 112	564

Fig. 4-17. Sequence alignment of the samples identified as *G. mosseae*. Bases not common among the samples were shaded with gray. The sequences shaded with black indicate *Hinf* I recognition sites (5'-GANTC-3').

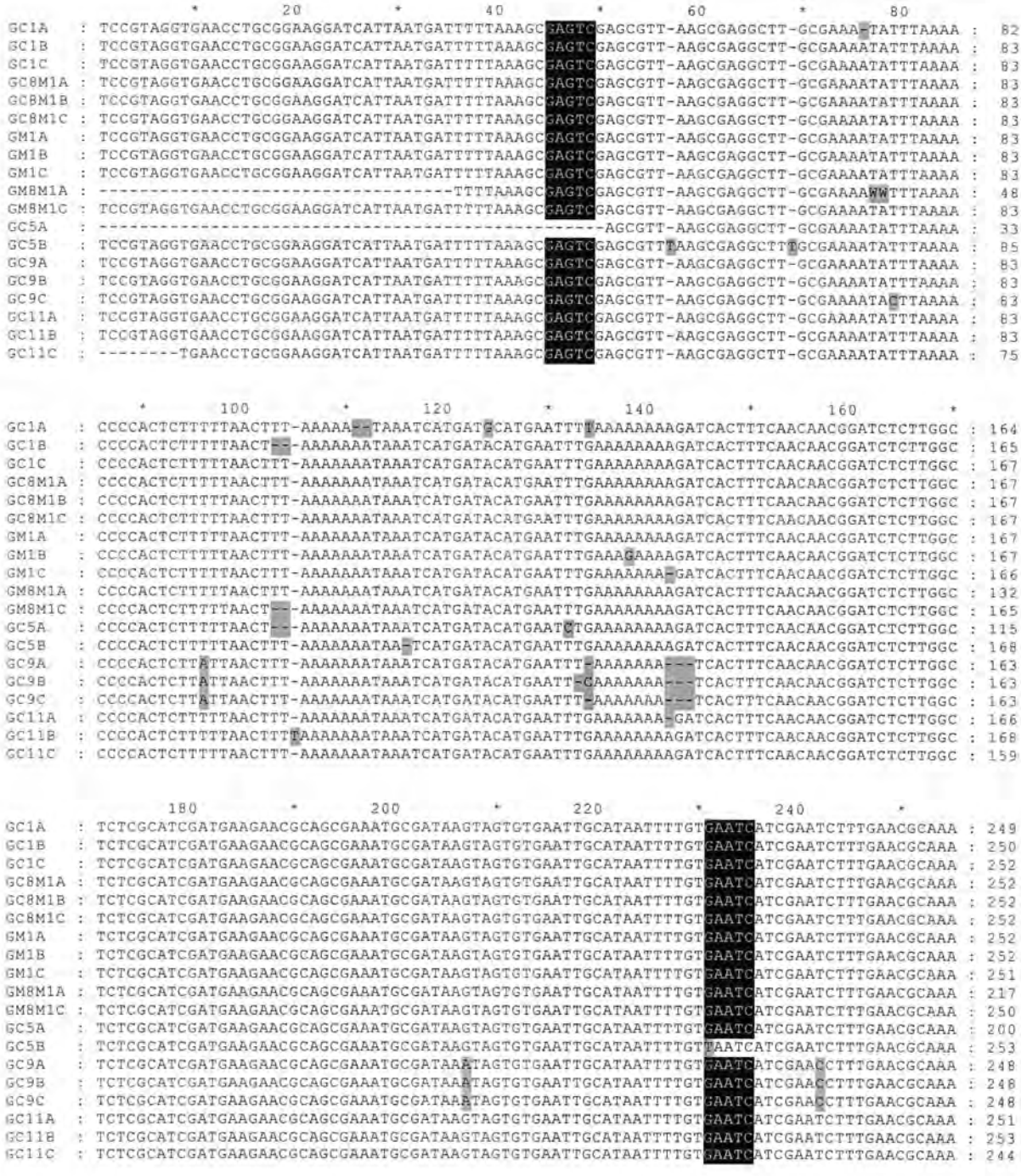


Fig. 4-17. (Continued)

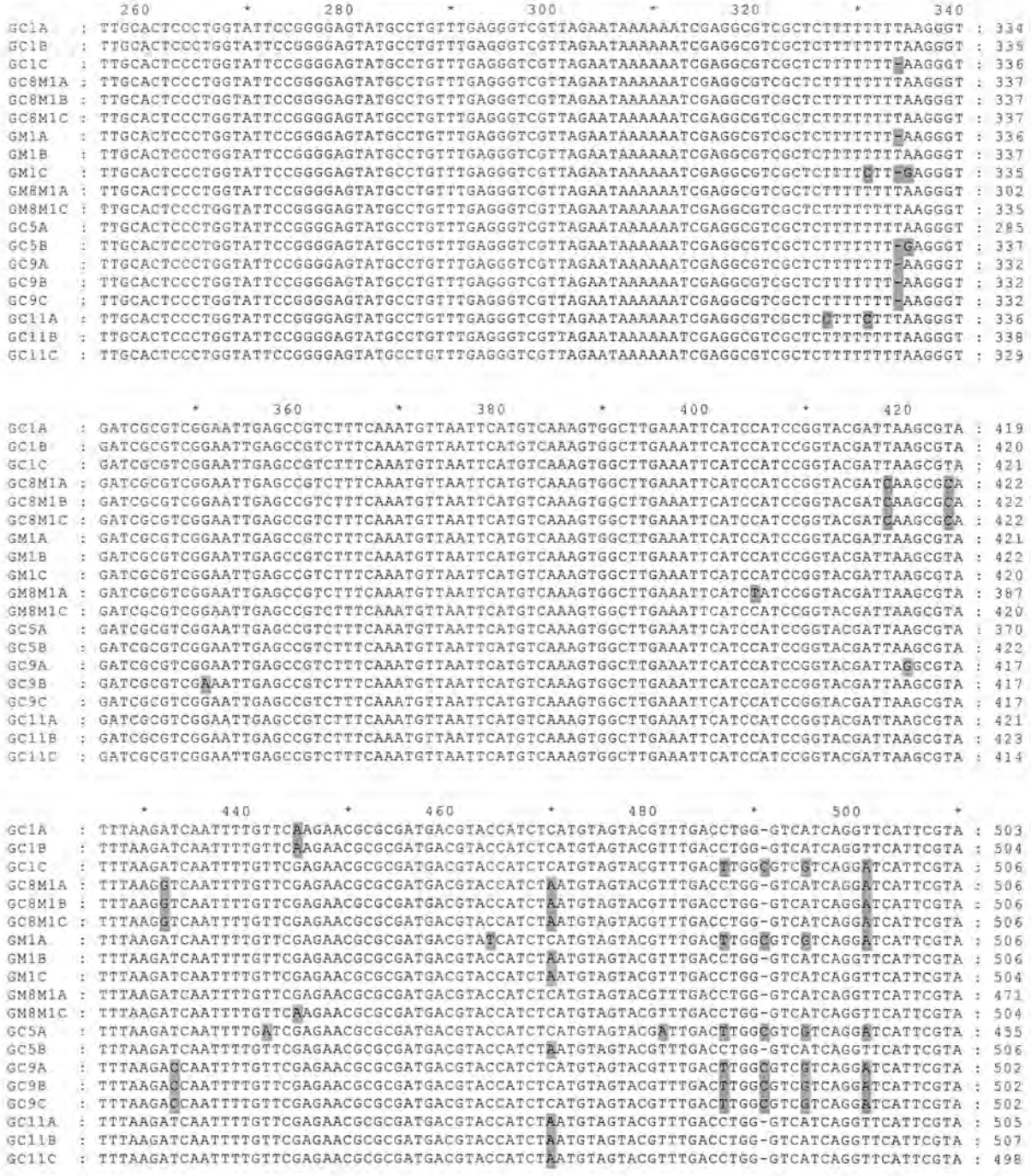


Fig. 4-17. (Continued)

```
                520         *       540         *       560         *       580
GC1A   : TACGATACTCAAA-CTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTNAGCATATCAATAAGCGGAGGA- : 577
GC1B   : TACGATACTCAAA-CTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 578
GC1C   : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 581
GC8M1A : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 581
GC8M1B : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 581
GC8M1C : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 581
GM1A   : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 581
GM1B   : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 581
GM1C   : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 579
GM8M1A : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 546
GM8M1C : TACGATACTCAAA-CTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 578
GC5A   : TACGATACTCAA--CTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 528
GC5B   : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 581
GC9A   : TACGATACTCNAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 577
GC9B   : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 577
GC9C   : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 577
GC11A  : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 580
GC11B  : TACGATACTCAAAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 582
GC11C  : TACGATACTCAAAACTTTTGGCCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA- : 573
```

Fig. 4-18. Sequence alignment of the samples identified as *G. caledonium*. Bases not common among the samples were shaded with gray. The sequences shaded with black indicate *Hinf* I recognition sites (5'-GANTC-3').

```
                *       20          *       40          *       60          *       80
GC7A : TCCGTAGGTGAACCTGCGGAAGGATCATTGATGATTTTTT-AAGCAATCCGAGCGAGATAAAGCGAGGATTGCGAAATATTTAAAAC :  86
GC7B : TCCGTAGGTGAACCTGCGGAAGGATCATTAATGATTTTTTTAAGCAATCCGAGCGAGATAAAGCGAGGATTGCGAAATATTTAAAAC :  87
GC7C : TCCGTAGGTGAACCTGCGGAAGGATCATTAATGATTTTTT-AAGCAATCTGAGCGAGATAAAGCGAGGATTGCGAAATATTTAAAAC :  86

          *       100         *       120         *       140         *       160         *
GC7A : CCCACTCTTTTTAACTTT-ATATAATAAT-AAATCATGATACATGAATTT-AAAAAAAA-GATCACTTTCAACAACGGATCTCTTGG : 169
GC7B : CCCACTCTTATTGACTTTTATATAATAATTAAATCATGATACATGAATTTTAAAAAAAAAGATCACTTTCAACAACGGATCTCTTGG : 174
GC7C : CCCACTCTTT--AACTTT-ATATAATAAT-AAATCATGATACATGAATTT-AAAAAAAA-GATCACTTTCAACAACGGATCTCTTGG : 167

            180         *       200         *       220         *       240         *       260
GC7A : CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAAATTGTGAATCATCGAATCTTTGAACGCAAATT : 256
GC7B : CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAAATTGTGAATCATCGAATCTTTGAACGCAAATT : 261
GC7C : CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAAATTGTGAATCATCGAATCTTTGAACGCAAATT : 254

                *       280         *       300         *       320         *       340
GC7A : GCACTCTCTGGTATTCCGGGGAGTATGCCTGTTTGAGGGTCGTTAGAACAAAAATCGAAGCGGTTGCTCTTTACGAAAT-ATTATTA : 342
GC7B : GCACTCTCTGGTATTCCGGGGAGTATGCCTGTTTGAGGGTCGTTAGAACAAAAATCGAAGC-GTCGCTCTTTACGAAATTATTAT-- : 345
GC7C : GCACTCTCTGGTATTCCGGGGAGTATGCCTGTTTGAGGGTCGTTAGAACAAAAATCGAAGC-GTCGCTCTTTACGAAATTATTAT-- : 338

          *       360         *       380         *       400         *       420         *
GC7A : TATTTTGTTTGGGTGATCGCGTCGGAATTGAACCGTCTTTCATATGTTAATTCATGTCAAAGTGGCTTAAAATTCATCAATCTGGTA : 429
GC7B : -ATTTTGTTTGGGTGATTGCGTCGGAATTGAGCCTTCTTTCATATGTTAATTCATGTCAAAGTGGCTTAAAATTCATCAATCTGGTA : 431
GC7C : -ATTTTGTTTGGGTGATTGCGTCGGAATTGAGCCTTCTTTCATATGTTAATTCATGTCAAAGTGGCTTAAAATTCATCAATCTGGTA : 424

            440         *       460         *       480         *       500         *       520
GC7A : CGATTTAAAGCGTATTT-AAGATCAACCTTGATTAAGAACGCGCGATGACGTACCATCTCATGTAGTACGTTCGACCTGCTTGTCAG : 515
GC7B : CGATTTAAAGCGTATTTTAAGATCAACCTTGATTAAGAACGCGCGATGACGTACCATCTCATGTAGTACGTTCGACCTGCTTGTCAG : 518
GC7C : CGATTTAAAGCGTATTT-AAGATCAACCTTGATTAAGAACGCGCGATGACGTACCATCTCATGTAGTACGTTCGACCTGCTTGTCAG : 510

                *       540         *       560         *       580         *       600
GC7A : GCTCATTCGTATACGACATTCAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA : 599
GC7B : GCTCATTCGTATACGACATTCAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA : 602
GC7C : GCTCATTCGTATACGACATTCAACTTTTGACCTCAAATCAGGTAAGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA : 594
```

Fig. 4-19. Sequence comparison among GC5C, *Gastrostyla steinii* (AF508758), *Oxytricha trifallax* (AJ286798), and *Oxytricha fallax* (AJ286797). Bases different from GC5C sequences are shaded with gray. The sequences shaded with black indicate *Hinf* I recognition sites (5'-GANTC-3').

```
                    *         20          *         40          *         60          *
GC5C        : TCCGTAGGTGAACCTGCGGAAGGATCATTAACACTAATCCACAACTACTCAACCAAGCCTAAAGTTGCAGCA :  72
Ga. steini  : tccgtaggtgaacctgcggaaggatcattaacactaatccacaactactcaaccaagcctaaagttgcagca :  72
O. trifall  : tccgtaggtgaacctgcggaaggatcattaacactaatccacaactactcaaccaagcctatagttgcagc- :  71
O. fallax   : tccgtaggtgaacctgcggaaggatcattaacactaatccacaactactcaaccaagcctatagttgtagca :  72

                  80          *         100         *         120         *         140
GC5C        : GTAGTACAGCCAGCGAAAGCAGG-CAGTGCGATGTTGCAACCTCAACAAAACTAATCAAACAAAGGACTCTA : 143
Ga. steini  : gtagtacagccagcgaaagcagg-cagtgcgatgttgcamcctcaacaaaactaatcaaacaaaggactcta : 143
O. trifall  : --agtacag-cagcgtaagcagg-cagtactgcgttgcaacctcaacaaaattaatcaaacaaagaactata : 139
O. fallax   : ttag-----ccagcgtaagcaggtcagtactgcgttgcaacctcaacaaaattaatcaaacaaagaactata : 139

                    *         160         *         180         *         200         *
GC5C        : ACTAAGTCAATCATCAAAACCAAATTCTCAACGATGGATATCTTGGTTCCCATTACGATGAAGAACGCAGCG : 215
Ga. steini  : aytaagtcaattatcaaaaccaaattctcaacgatggatatcttggttcccattacgatgaagaacgcagcg : 215
O. trifall  : actaagtcaatcatcaaaaccaaattctcaacgatggatatcttggttcctattacgatgaagaacgcagcg : 211
O. fallax   : actaagtcaatcatcaaaaccaaattctcaacgatggatatcttggttcctattacgatgaagaacgcagcg : 211

                  220         *         240         *         260         *         280
GC5C        : AAGTGCGATAAGCAATGCGAATTGCAGAACCGTGAGTCATCAGATTTTTGAACGCAACTGGCGCTAGGTGGT : 287
Ga. steini  : aagtgcgataagcaatgcgaattgcagaaccgtgagtcatcagatttttgaacgcaactggcgctaggtggt : 287
O. trifall  : aagtgcgataagcaatgcgaattgcagaaccgtgagtcatcagatttttgaacgcaactggcgctagaaggt : 283
O. fallax   : aagtgcgataagcaatgcgaattgcagaaccgtgagtcatcagatttttgaacgcaactggcgctagaaggt : 283

                    *         300         *         320         *         340         *         360
GC5C        : TATCCACCTAGCATGCTTGTTTCAGTGTCTTTTTATTTCCTAACCGAAATCATAATGCGAGAGATACCCTTC : 359
Ga. steini  : tatccacctagcatgcttgtttcagtgtctgtttatttcctaaccgaaatcataatgcgagagatacccttc : 359
O. trifall  : tatccttttagcatgcttgtttcagtgtcttttttatttcctaaccgaaatcataatgcgagagatatccttc : 355
O. fallax   : tatccttttagcatgattgtttcagtgtcttttttatttcctaaccgaaatcataatgcgagagatatccttc : 355

                    *         380         *         400         *         420         *
GC5C        : TCTTGTTAAGCATGAAAGCACTCTGCGCTCTGCGAGCGGCTTCGGT----------CGCACTCAATGCAGCA : 421
Ga. steini  : tcttgttaagcatgaaagcactctgcgctctgcgagcggcttcggt----------cgcactcaatgcagca : 421
O. trifall  : tcttgttaagcatgaaagcactctgcgctctgcgagcggcttcgattcgtcagggtcgcactcaatgcagca : 427
O. fallax   : tcttgttaagcatgaaagcactctgcgctctgcgagcggcttcgattcgtcagggtcgcactcaatgcagca : 427

                  440         *         460         *         480         *         500
GC5C        : GTCACATTGTTTACAGTGTGAACTCATTGAGAGTAGAGGCAGTGCGGTGTAACAACTGCCAGTGCTTAGACA : 493
Ga. steini  : gtcacattgtttacagtgtgaactcattgagagtagaggcagtgcggtgtaacaactgccagtgcttagaca : 493
O. trifall  : gtcacattgtttacagtgtgaactcattgagagtagaggcagtgcggtgtaacaactgccagtgcttagaca : 499
O. fallax   : gtcacattgtttacagtgtgaactcattgagagtagaggcagtgcggtgtaacaactgccagtgcttagaca : 499

                    *         520         *         540         *         560         *
GC5C        : CTCAAACTTTCTTTGTATCTGAAATCAAGCAGGATCACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA : 564
Ga. steini  : ctcaaacttttcttgtatctgaaatcaagcaggatcacccgctgaacttaagcatatcactaagcggagga : 564
O. trifall  : ctcaaacttttcttgtatctgaaatcaagcaggatcacccgctgaacttaagcatatcaataagcg----- : 565
O. fallax   : ctcaaacttttcttgtatctgaaatcaagcaggatcacccgctgaacttaagcatatcactaagcg----- : 565
```

The sequence of GM8M1B was identical with luciferase gene (97.3%) and did not contain the pGEM-T sequences, indicating a possible contamination outside the PCR and cloning process. Due to relatively poor sequence results, only reliable sequences are shown in GC8M1A although full length of PCR product (581 bp) was inserted (Fig. 4-15). Shorter sequences (528 and 573 bp) than full length of PCR products were inserted in GC5A and GC11C, respectively (Fig. 4-15 and 4-17).

Discussion

Soybean growth and effect of AM fungi

Four wk were not long enough to observe differences in shoot dry weight (Fig. 4-2 and 4-3). Although shoot dry weight of BSR201 was different among the treatments after 8-wk growth, we did not observe a significant increase in shoot dry weight with the inoculation of AM fungi compared with the control (Fig. 4-4 and 4-5). SC had a tendency to decrease the shoot dry weight compared with control, although this decrease was not significant. Pair-wise comparison showed that the shoot dry weight of BSR201 in SC treatment was significantly different from that in GCGM treatment, suggesting different AM fungal treatment provided different impact on soybean growth.

Poor ability of SC as a mutualistic symbiont also has been documented by several authors when SC was associated with subterranean clover (*Trifoloum subterraneum* L.) (Abbott and Robson, 1985; Thomson et al., 1986, 1990; Jakobsen et al., 1992a) and with cucumber (*Cucumis sativus* L.) (Pearson and Jakobsen, 1993a). Earlier work using ^{32}P

showed that SC transported much less P to subterranean clover compared with *Acaulospora laevis* (Gerd. & Trappe) and *G. invermanium* Hall (Jakobsen et al., 1992b) and to cucumber compared with *G. caledonium* and *G. invermanium* (Pearson and Jakobsen, 1993a, b). *S. calospora* (WUM12) was reported to accumulate P in its hyphae (Jakobsen et al., 1992b). Also, SC was reported to require more carbohydrate than *Glomus sp.* and *G. caledonium* (Pearson & Jakobsen, 1993a).

Positive effects of SC on plant growth have also been reported when SC was associated with wheat (*Triticum aestivum* L.) (Graham & Abbott, 2000) and with Barrelclover (*Medicago truncatula* L.) (Smith et al., 2000).

Existence of both positive and negative interactions between SC and its host plants might suggest the concept of preferable host-fungal combinations based on functional complementarity/redundancy (Koide, 2000). According to this concept, our result indicated that soybean roots did not complementarily function with the hyphae of SC.

Several factors such as developmental, environmental, and genotypic factors could affect AM fungi to be negative or positive (Johnson et al., 1997). AM fungal colonization can decrease the growth of plant seedlings in the few wk after planting (Abbott and Robson, 1985; Koide, 1985; Thomson et al., 1986, 1990; Jakobsen et al., 1992a; Johnson et al., 1997). *S. calospora* colonized relatively slowly, and this slowness might explain the negative effects of AM fungi on their host plants by requiring more carbohydrate for a longer time than AM fungi that colonize faster (Hart and Reader, 2002).

AM fungal colonization in roots

Mycorrhizal colonization rates 8 wk after planting were significantly different among treatments both in BSR201 and Mandarin (Fig. 4-6 and 4-7). Colonization rates in both BSR201 and Mandarin were significantly different among three AM fungal species, GC, GM, and SC, although GC was likely to be contaminated with *G. mosseae* (discussed later). The AM fungi colonizing in GM and SC treatments were identified as *G. mosseae* (lane 11-13 of Fig. 4-13) and *S. calospora* (lane 9 of Fig. 4-11), respectively, and no contamination was detected. Although similar tendencies in mycorrhizal colonization rates among the treatments were obtained both from BSR201 and Mandarin, colonization rates in BSR201 were approximately 1.3 times higher than those in Mandarin. Differences in mycorrhizal colonization rates between BSR201 and Mandarin were discussed in Chapter 3.

Colonization rates of SC were extremely low in both BSR201 and Mandarin (Fig. 4-6 and 4-7). Although *S. calospora* is generally a slow grower compared with *Glomaceae*, this fungus begins to infect around 4 wk after inoculation (Hart and Reader, 2002); therefore, 8-wk growth in this study should be enough time to observe mycorrhizal colonization.

There are several possible explanations for the low colonization of SC. First, growth conditions might not be adequate for this fungus. SC (*Scutellospora calospora* WUM 12) was isolated from a bushland community in Badgingara, Western Australia, in which soil pH was 4.8-5.5 and soil P level was less than 5 ppm. The soil used in our study had pH value of 6.9 and higher P level (38 ppm) with fine-loamy properties. Soil pH and P concentration largely affect colonization of AM fungi (Smith and Read, 1997). The poor drainage of our soil is probably different from the soil in the semiarid area where SC was isolated.

Second, the SC inoculum provided from the INVAM was a little old. It had been cultured over 12 cycles and stored for more than two years since 12th culture cycle. This isolate also is reported to grow and reproduce inconsistently in pot culture (Morton, personal communication). Therefore, its spore viability might be low. *Gigasporaceae* including *S. calospora* infect mostly from spores and rarely from mycorrhizal roots (Klironomos and Hart, 2002), supporting the idea that low number of viable spores could result in low performance in colonization.

Third, colonization strategy is different between *Gigasporaceae* including *S. calospora* and *Glomaceae* including *G. mosseae* and *G. caledonium* (Table 4-1; Hart and Reader, 2002; Klironomos and Hart, 2002). Although *Glomaceae* grow faster and colonize plant roots more than *Gigasporaceae*, they have shorter hyphal length and less fungal biomass in soil. Hart and Reader (2002) suggested that the colonization rates in plant roots are biased measurements of AM fungi because of these differences. We measured only colonization within plant roots, but both internal and external measurement of colonization might be necessary to overcome this problem.

Fourth and lastly, plant-resistant responses might work against SC stronger than GC and GM. This means that SC was not as effective as GC and GM to suppress the plant-resistant responses or SC might be good at inducing these responses.

More research is necessary to clarify the poor ability of SC to colonize in roots.

Contaminant in GC inoculum

Because of low colonization in SC treatments, we concentrated on the interaction between GC and GM. Another problem, however, faced us. RFLP and sequencing results

suggested that the GC inoculum was contaminated with *G. mossseae* (Fig. 4-13, 4-14, 4-16, Table 4-5). Contamination with undescribed *Glomus sp.* in *G. caledonium* RIS42 inoculum was also reported by Dr. Joe Morton at the INVAM (personal communication). Based on our sequencing results, the contaminant found in our study was identical with *Glomus mosseae* (Table 4-5), and it was very close to GM (Fig. 4-17, Table 4-8). Similarity among the sequences identified as *G. mosseae* were very high (>97%), and in some case, it was 100% (among GC8M1 and between GC1B and GM8M1C). *G. mosseae* BEG83 (GC) was contributed by the same group who had used GC (*G. caledonium* RIS42). We are requesting more information about GM from Dr. Søren Rosendahl at the University of Copenhagen, who isolated this strain. Since spores of *G. caledonium* look similar to those of *G. mosseae* under a dissecting microscope (INVAM online, 2003), the contaminant (*G. mosseae*) might not be removed completely by evaluating spore morphology. The contaminant information obtained in this study was reported to the INVAM.

Our results suggested that the contaminant identified as *G. mosseae* colonized more aggressively than *G. caledonium*. Also, more spores looked like *G. mosseae* (small and brown spores) than *G. caledonium* (big and brown spores) after the initial propagation in the greenhouse for 10 wk (data not shown). This observation was compatible with the comment by Dr. Joe Morton that yield of sporulation by *G. caledonium* has never been very good (personal communication).

Although there were three RFLP patterns in Fig. 4-14, we only found two AM fungi, *G. caledonium* and *G. mosseae*, based on the sequencing results (Table 4-5). The third RFLP pattern (group C in Table 4-4) was derived from both *G. mosseae* and *Gastrostyla steinii* (Fig. 4-14 and Table 4-7). Since *Ga. steinii* has a large body (150-320 μm in length),

Table 4-8. Similarity matrix (%) among the sequences identified as *G. mosseae*. Approximately 580 bp were compared.

		GC1			GC8M1			GM1			GM8M1		GC5		GC9			GC11		
		A	B	C	A	B	C	A	B	C	A	C	A	B	A	B	C	A	B	C
GC1	A	100.0	99.0	97.9	97.8	97.8	97.8	97.8	98.3	98.1	98.0	99.0	97.5	97.8	97.1	97.1	97.2	98.1	98.3	98.3
	B		100.0	98.6	98.5	98.5	98.5	98.5	99.0	98.8	98.7	100.0	98.5	98.5	97.4	97.4	97.6	98.8	99.0	99.0
	C			100.0	98.8	98.8	98.8	98.8	99.0	98.6	98.7	98.6	98.7	98.6	98.3	98.3	98.5	98.6	99.1	97.6
GC8M1	A				100.0	100.0	100.0	98.6	99.1	98.6	98.5	99.0	97.6	98.6	97.1	97.1	97.2	98.8	99.3	97.8
	B					100.0	100.0	98.6	99.1	98.6	98.5	99.0	97.6	98.6	97.1	97.1	97.2	98.8	99.3	97.8
	C						100.0	98.6	99.1	98.6	98.5	99.0	97.6	98.6	97.1	97.1	97.2	98.8	99.3	97.8
GM1	A							100.0	98.8	98.5	98.5	98.5	98.5	98.5	98.1	98.1	98.3	98.5	99.0	97.4
	B								100.0	99.1	99.1	99.0	97.7	99.1	97.2	97.2	97.4	99.3	99.8	99.7
	C									100.0	98.5	98.5	97.5	99.1	97.1	97.7	97.6	99.5	99.1	99.1
GM8M1	A										100.0	98.7	97.6	98.5	96.9	96.9	97.1	98.7	99.1	99.1
	C											100.0	98.5	99.0	97.4	97.4	97.6	99.1	99.7	99.0
GC5	A												100.0	97.2	97.3	97.3	97.5	97.5	97.7	97.7
	B													100.0	96.9	96.9	97.1	98.8	99.3	99.1
GC9	A														100.0	99.3	99.5	97.2	97.3	97.2
	B															100.0	99.5	97.2	97.3	97.2
	C																100.0	97.4	97.4	98.3
GC11	A																	100.0	99.7	99.3
	B																		100.0	99.8
	C																			100.0

this organism is not likely to live inside the AM fungal spores. The cysts of *Ga. steinii* are brown and globular like the spores of *G. caledonium* and *G. mosseae*; therefore, we might have selected them by accident, although we worked with Zahra Troeh, who has a lot of experiences in spore extraction. Since even cleaned single spores contain plant, animal, or ascomycetes DNA (Schüßler, 1999), *Ga. steinii* DNA might have contaminated the spores of *G. mosseae*. Detection of both *Ga. steinii* and *G. mosseae* is still questionable. Because *Ga. steinii* also belongs to Eukaryotes, its DNA was amplified with ITS1 and ITS4. In order to avoid unexpected amplification, use of fungal specific primer such as ITS1F (Gardes and Bruns, 1993) is necessary.

The sequence of GM8M1B was identical with luciferase gene. Luciferase is one of the florescent proteins produced by firefly (*Luciola sp.*) and Sea pansy (*Renilla reniformis*). Many plasmid vectors contain this gene to measure the activity of promoters of interest (reporter assay). Since we have never used this gene and the vector containing this gene, this sample might be contaminated during the sequence reaction. We reported this problem to the DNA sequencing and synthesis facility, Iowa State University, Ames, IA.

Insertion of shorter sequences than full length of PCR products in GC5A and GC11C indicated that some of the PCR products might be physically destroyed by freezing and thawing because they were stored at -20°C prior to transformation and cloning.

Conclusion

Based on the information we obtained in this study and on the comments provided from other researchers, we concluded as follows: first, the GC (*G. caledonium* RIS42) inoculum was contaminated with *G. mosseae*; second, the partial rRNA sequences of the

contaminant *G. mosseae* was very close to GM (*G. mosseae* BEG83); third, the contaminant *G. mosseae* colonized more within the roots and produced more spores than *G. caledonium*, suggesting the competitiveness of *G. mosseae* against *G. caledonium*.

Our initial model using three AM fungi did not function as expected because of the contamination in GC inoculum and the low colonization activity of SC; however, we found that AM fungal effects on soybean growth were different among fungal inocula. Low colonization rates in SC treatment and somewhat negative effect of SC were observed in this study. More research is necessary on the interaction between SC and the host plants to verify the concept of functional complementarity/redundancy between roots and hyphae and preferable host-fungus combinations (Koide, 2000). In GC inoculum, competition might exist because *G. caledonium* seemed to be excluded from the roots by the contaminant identified as *G. mosseae*. The contaminant in the inoculum of GC (*G. caledonium* RIS42) should be eliminated for reliable research.

References

Abbott, L.K., and A.D. Robson. 1984. Colonization of the root system of subterranean clover by three species of vesicular-arbuscular mycorrhizal fungi. New Phytol. 96:275-281.

Adelman, M.J., and J.B. Morton. 1986. Infectivity of vesicular-arbuscular mycorrhizal fungi: Influence of host-soil diluent combinations on MPN estimates and percentage colonization. Soil Biol. Biochem. 18:77-83.

Brundrett, M., N. Bougher, B. Dell, T. Grove, and N. Malajczuk. 1996. Working with mycorrhizas in forestry and agriculture. Australian Centre for International Agricultural Rresearch, Canberra.

Clapp, J.P., J.P.W. Young, J.W. Merryweather, and A.H. Fitter. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol. 130:259-265.

Daniell, T.J., R. Husband, A.H. Fitter, and J.P.W. Young. 2001. Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbial Ecol. 36:203-209.

Gardes, M., and T.D. Brund. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-118.

Giovannetti, M., and B. Mosse. 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84:489-500.

Graham, J.H., and L.K. Abbott. 2000. Wheat responses to aggressive and non-aggressive arbuscular mycorrhizal fungi. Plant Soil 220:207-218.

Hart, M.M., and R.J. Reader. 2002. Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol. 153:335-344.

Helgason, T., T.J. Daniell, R. Husband, A.H. Fitter, and J.P.W. Young. 1998. Ploughing up the wood-wide web? Nature (London) 394:431.

Helgason, T., A.H. Fitter, and J.P.W. Young. 1999. Molecular diversity of arbuscular mycorrhizal fungi colonizing *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. Mol. Ecol. 8:659-666.

Hepper, C.M., C. Azcon-Aguilar, S. Rosendahl, and R. Sen. 1988. Competition between three species of *Glomus* used as spatially separated introduced and indigenous mycorrhizal inocula for leek (*Allium porrum* L.). New Phytol. 110:207-215.

INVAM. 2003. Species Diagnosis Pages [Online]. Available at http://invam.caf.wvu.edu/fungi/taxonomy/speciesID.htm (verified 20 July 2003)

Jacquot, E., D. van Tuinen, S. Gianinazzi, and V. Gianinazzi-Pearson. 2000. Monitoring species of arbuscular mycorrhizal fungi *in planta* and in soil by nested PCR: application to the study of the impact of sewage sludge. Plant Soil 226:179-188.

Jakobsen, I., L.K. Abbott, and A.D. Robson. 1992a. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L.: 1. Spread of hyphae and phosphorus inflow into roots. New Phytol. 120:371-380.

Jakobsen, I., L.K. Abbott, and A.D. Robson. 1992b. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L.: 2. Hyphal transport of ^{32}P over definded distances. New Phytol. 120:509-516.

Johnson, N.C., J.H. Graham, and F.A. Smith. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol. 135:575-585.

Khalil, S., T.E. Loynachan, and M.A. Tabatabai. 1994. Mycorrhizal dependency and nutrient uptake by improved and unimproved corn and soybean cultivars. Agron. J. 86:949-958.

Klironomos, J.K., and M.M. Hart. 2002. Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza 12:181-184.

Koide, R.T. 2000. Functional complementarity in the arbuscular mycorrhizal symbiosis. New Phytol. 147:233-235.

Lopez-Aguillon, R., and B. Mosse. 1987. Experiments on competitiveness of three endomycorrhizal fungi. Plant Soil 97:155-170.

Moorman, T., and F.B. Reeves. 1979. The role of endomycorrhizae in revegetation practices in the semi-arid west. II. A bioassay to determine the effect of land disturbance on endomycorrhizal populations. Amer. J. of Bot. 66:14-18.

Pearson, J.N., and I. Jakobsen. 1993. Symbiotic exchange of carbon and phosphorus between cucumber and three arbuscular mycorrhizal fungi. New Phytol. 124:481-488.

Pearson, J.N., and I. Jakobsen. 1993. The relative contribution of hyphae and roots to phorphorus uptake by arbuscular mycorrhizal plants, measured by dual labelling with ^{32}P and ^{33}P. New Phytol. 124:489-494.

Redecker, D. 2000. Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10: 73-89.

Sainz, M.J., A. Vilariño, and J. Arisones. 1989. Competition between *Glomus tenue* and some coarse fungi for colonizing red clover roots in acid soils. Agric. Ecosyst. Environ. 29:337-340.

Smith, F.A., I. Jakobsen, and S.E. Smith. 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol. 147:357-366.

Smith, S., and D. Read. 1997. Mycorrhizal symbiosis. 2nd Edn. Academic Press, London,

Streitwolf-Engel, R., T. Boller, A. Wiemken, and I.R. Sanders. 1997. Clonal growth traits of two *Prunella* species are determined by co-occuring arbuscular mycorrhizal fungi from a calcareous grassland. J. Ecol. 85:181-191.

Thomson, B.D., A.D. Robson, and L.K. Abbott. 1986. Effects of phosphorus on the formation of mycorrhizas by *Gigaspora calospora* and *Glomus fasciculatum* in relation to root carbohydrates. New Phytol. 103:751-765.

Thomson, B.D., A.D. Robson, and L.K. Abbott. 1990. Mycorrhizas formed by *Gigaspora calospora* and *Glomus fasciculatum* on subterranean clover in relation to carbohydrate concentration in roots. New Phytol. 114:217-225.

Troeh, Z., and T.E. Loynachan. 2003. Endomycorrhizal fungal survival in continuous corn, soybean, and fallow. Agron. J. 95: 224-230

van der Heijden, M.G.A., J.K. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I.R. Sanders. 1998a. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature (London) 396:69-72.

van der Heijden, M.G.A., T. Boller, A. Wiemken, and I.R. Sanders. 1998b. Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082-2091.

van der Heijden, M.G.A., A. Wiemken, and I.R. Sanders. 2002. Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occuring plant. New Phytol. 157:569-578.

van Tuinen, D., E. Jacquot, B. Zhao, A. Gollotte, and V. Gianinazzi-Pearson. 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol. Ecol. 7:879-887.

Vandenkoornhuyse, P., R. Husband, T.J. Daniell, I.J. Watson, J.M. Duck, A.H. Fitter, and J.P.W. Young. 2002. Arbuscular mycorrhizal community composition assoiciated with two plant species in a grassland ecosystem. FEMS Microbial Ecol. 11:1555-1564.

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315-322. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (ed.) PCR protocols, a guide to methods and applications. Academic Press, London.

Wilson, J.M., and M.J. Trinick. 1983. Infection development and interactions between vesicular-arbuscular mycorrhizal fungi. New Phytol. 93:543-553.

Wilson, J.M. 1984. Competition for infection between vesicular-arbuscular mycorrhizal fungi. New Phytol. 97:427-435.

Appendix

Table 4A. Raw data for Fig. 4-1 and 4-2. Shoot dry weight of soybean cv. BSR201 and Mandarin 4 wk after planting.

BSR201				Mandarin			
GCGM	GCSC	GMSC	Control	GCGM	GCSC	GMSC	Control
g							
0.69	0.48	0.32	0.55	0.84	0.77	0.38	0.62
0.71	0.56	0.56	0.71	0.76	0.18	0.68	0.45
0.55	0.69	1.18	0.21	0.97	0.56	1.18	0.57
1.09	0.64	0.47	0.46	0.13	0.57	0.47	0.48
0.57	0.68	0.76	0.22	0.79	1.08	0.76	0.39
ND†	0.71	0.78	0.64	ND†	0.89	0.78	1.11

†ND, not determined.

Table 4B. Raw data for Fig. 4-4. Shoot dry weight of soybean cv. BSR201 8 wk after planting.

GC	GM	SC	GCGM	GCSC	GMSC	GCGMSC	Control
g							
1.67	2.41	1.22	3.87	0.98	1.62	3.35	2.16
2.74	1.31	0.73	1.32	0.74	2.37	1.61	1.85
2.68	3.11	0.42	2.60	1.41	2.17	2.11	0.86
3.64	3.99	0.93	3.50	1.14	2.65	1.97	0.96
	1.77		3.10	2.56	2.01	2.62	2.09
	3.75		2.49	2.37	1.68	3.00	1.75

Table 4C. Raw data for Fig. 4-5. Shoot dry weight of soybean cv. Mandarin 8 wk after planting.

GC	GM	SC	GCGM	GCSC	GMSC	GCGMSC	Control
g							
2.43	1.44	1.35	2.49	2.41	2.07	2.71	1.89
1.97	2.36	2.06	2.47	3.19	3.23	2.59	2.19
2.93	2.79	1.54	3	2.26	2.62	2.67	2.99
2.72	2.23	1.51	2.15	1.07	2.7	1.21	2.59
	2.54		1.31	2	1.55	3.15	2.26
	2.79		2.03	2.27	2.53	2.46	0.86

Table 4D. Raw data for Fig. 4-6. Mycorrhizal colonization rates in soybean cv. BSR201 8 wk after planting.

	GC			GM			SC			GCGM			GCSC			GMSC			Control		
rep.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	29	26	23	53	55	52	0	1	0	42	43	41	30	31	29	40	35	30	0	0	0
	43	30	36	55	57	55	0	0	0	40	43	40	31	28	33	41	35	36	0	0	0
	47	43	39	50	48	55	0	0	0	41	35	34		ND†			ND†		0	0	0
	37	41	52	48	49	50	0	0	1	34	34	36		ND†			ND†		0	0	0
				51	52	52				45	43	41		ND†			ND†		0	0	0
				58	53	52				35	36	43		ND†			ND†		0	0	0

†ND, not determined.

Table 4E. Raw data for Fig. 4-7. Mycorrhizal colonization rates in soybean cv. Mandarin 8 wk after planting.

	GC			GM			SC			GCGM			GCSC			GMSC			Control		
rep.	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	37	34	35	45	42	42	0	0	0	29	32	35	26	20	19	26	28	29	0	0	0
	25	33	49	37	35	39	0	1	0	27	25	26	18	18	17	25	31	23	0	0	0
	23	18	22	44	41	37	0	2	1	32	33	33	28	25	25	27	25	28	0	0	0
	28	27	24	41	42	38	0	2	3	25	30	27	21	15	22	30	26	32	0	0	0
				47	45	43				35	34	34		ND†		34	33	33	0	0	0
				38	37	35				30	32	30		ND†		29	28	27	0	0	0

†ND, not determined.

Acknowledgment

I would like to express my greatest appreciation and gratitude to my major professor, Dr. Thomas E. Loynachan, for his thoughtful advice and support throughout my study at Iowa State University. I would also like to thank to Drs. Larry J. Halverson and Thomas C. Harrington, the members of my committee, for their advice and support.

I would like to acknowledge Dr. Gwyn Beattie, Plant Pathology at Iowa State University, and Dr. Roger T. Koide, Horticulture at Pennsilvania State University, for their helpful suggestions and encouragement. My gratitude would be expressed to Dr. Joseph B. Morton at International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) for providing AM fungal inocula and providing useful suggestions.

I would like to thank to Dr. Michael Thompson, Agronomy at Iowa State University, for providing us access to his facilities. All the people who helped me with my work should be gratfully acknowledged, including Woo-Suk Chang, Zahra Troeh, Martijn van de Mortel, and other lab mates. Many thanks to the scientific and administrative staffs for supporting this research and my program of study at Iowa State University.

Last, I would like to thank my parents, Akira and Kayoko Ishii, for their great understanding, concern, patience, and love.